Licensing of myeloid cells promotes central nervous system autoimmunity and is controlled by peroxisome proliferator-activated receptor γ

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During central nervous system autoimmunity, interactions between infiltrating immune cells and brain-resident cells are critical for disease progression and ultimately organ damage. Here, we demonstrate that local cross-talk between invading autoreactive T cells and auto-antigen-presenting myeloid cells within the central nervous system results in myeloid cell activation, which is crucial for disease progression during experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis. This T cell-mediated licensing of central nervous system myeloid cells triggered astrocytic CCL2-release and promoted recruitment of inflammatory CCR2⁺-monocytes, which are the main effectors of disease progression. By employing a cell-specific knockout model, we identify the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) in myeloid cells as key regulator of their disease-determining interactions with autoreactive T cells and brain-resident cells, respectively. LysM-PPARγKO mice exhibited disease exacerbation during the effector phase of experimental autoimmune encephalomyelitis characterized by enhanced activation of central nervous system myeloid cells accompanied by pronounced local CCL2 production and inflammatory monocyte invasion, which finally resulted in increased demyelination and neuronal damage. Pharmacological PPARγ activation decreased antigen-specific T cell-mediated licensing of central nervous system myeloid cells, reduced myeloid cell-mediated neurotoxicity and hence dampened central nervous system autoimmunity. Importantly, human monocytes derived from patients with multiple sclerosis clearly responded to PPARγ-mediated control of proinflammatory activation and production of neurotoxic mediators. Furthermore, PPARγ in human monocytes restricted their capacity to activate human astrocytes leading...
to dampened astrocytic CCL2 production. Together, interference with the disease-promoting cross-talk between central nervous system myeloid cells, autoreactive T cells and brain-resident cells represents a novel therapeutic approach that limits disease progression and lesion development during ongoing central nervous system autoimmunity.

Keywords: EAE; multiple sclerosis; inflammatory monocytes; PPAR
Abbreviations: EAE = experimental autoimmune encephalomyelitis; ELISA = enzyme-linked immunosorbent assay; KO = knockout; LPS = lipopolysaccharide; MOG = myelin oligodendrocyte glycoprotein; PPARγ = peroxisome proliferator-activated receptor γ; TNFα = tumour necrosis factor α; WT = wild-type

Introduction

The central role of autoreactive encephalitogenic CD4+ T cells has clearly been demonstrated in the animal model of multiple sclerosis experimental autoimmune encephalomyelitis (EAE) as CNS autoimmunity can be elicited by adoptive transfer of autoreactive myelin oligodendrocyte glycoprotein (MOG)-specific CD4+ T cells (Stinisson et al., 1998). These autoreactive CD4+ T cells are primed in the periphery within lymphatic tissues, migrate across the blood–brain barrier, and initiate autoimmune inflammation upon local reactivation by antigen-presenting cells (Reboldi et al., 2009). Besides encephalitogenic T cells, myeloid cells are recruited into the CNS and can outnumber lymphocytes in acute multiple sclerosis lesions by at least 10–20 times (Prineas and Graham, 1981; Barnett et al., 2006). During the last years, it became evident that CNS-infiltrating myeloid cells substantially contribute to local inflammatory processes as well as demyelination and axonal damage in multiple sclerosis and EAE (Geissmann et al., 2003; Lassmann, 2004; Mildner et al., 2009). Recent studies further revealed that the subpopulation of inflammatory CCR2+Ly-6Chi monocytes (Geissmann et al., 2003) amplifies the effector phase of CNS autoimmunity (Mildner et al., 2009) and that in vivo depletion of this subpopulation reduces disease severity (Tran et al., 1998). Moreover, CNS-resident microglial cells can also contribute to CNS inflammation during EAE (Heppner et al., 2005). However, the mechanisms driving myeloid cell activation during sterile autoimmunity induced by autoreactive T cells have not been resolved. Similarly, the cell-intrinsic factors that regulate responsiveness of myeloid cells towards such activation remained elusive.

A potent regulator of macrophage activation is the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), a ligand-activated transcription factor that mediates a broad range of anti-inflammatory effects (Ricote et al., 1998; Clark et al., 2000; Storer et al., 2005; Klotz et al., 2007). PPARγ in myeloid cells acts by interference with NFκB-target genes through a transrepression mechanism mediated by ligand-induced stabilization of the NCoR co-repressor complex at the promoter sites of pro-inflammatory genes such as inducible nitric oxide synthase or tumour necrosis factor α (TNFα) (Glass and Ogawa, 2006; Straus and Glass, 2007).

Interestingly, peripheral blood mononuclear cells from patients with multiple sclerosis exhibited strongly decreased PPARγ expression levels when compared with healthy controls (Klotz et al., 2005). As we have previously shown that ablation of PPARγ expression in dendritic cells increases their capacity to prime naïve CD4+ T cells (Klotz et al., 2007), we reasoned that PPARγ may serve as a cell-intrinsic regulator of myeloid cell activity, and hence, that diminished PPARγ activity in myeloid cells may critically contribute to CNS autoimmunity. In this study, we therefore addressed the question whether PPARγ limits myeloid cell activation during CNS autoimmunity and thus restricts local innate immune responses during autoimmune inflammation in the CNS.

Materials and methods

Mice

Mice were bred and maintained under specific pathogen-free conditions at the Haus für Experimentelle Therapie, University of Bonn and used for experiments at 7–12 weeks of age. C57BL/6 mice and congenic C57BL/6 (CD45.1) mice were purchased from Charles River Laboratories. PPARγfl/fl mice were obtained from Jackson Laboratories. Mice with a conditional PPARγ knockout in myeloid cells (i.e. LysM-PPARγKO mice) were generated by crossing PPARγfl/fl mice with LysM-Cre transgenic mice, which express the Cre recombinase under control of the murine LysM lymphocyte gene in monocytes/macrophages, microglia and neutrophils (Clausen et al., 1999) (Supplementary Fig. 1). To analyse recombination efficiency, LysM-Cre mice were crossed to R26-eYFPfl/wt mice (Fig. 1A and Supplementary Fig. 1A). Mice with a conditional knockout of PPARγ in CNS-resident cells of neuroectodermal origin, i.e. neurons, astrocytes and oligodendrocytes, were generated by crossing PPARγfl/fl mice with Nestin-Cre transgenic mice (Tronche et al., 1999) (Supplementary Fig. 3). Lines were backcrossed onto the C57BL/6 background for at least 10 generations. Genotyping of mice was performed by amplifying a portion of the particular gene from tail DNA. 5’-TGAATGGAAAG-3’ and 5’-TGTTTCCATCCATAAGTT-3’ primers were used to detect truncated or wild-type form of PPARγ gene in PPARγfl/fl mice. Genotyping of Cre recombinase in LysM-PPARγKO mice was performed using the primers 5’-TGTTTCCATCCATAAGTT-3’ and 5’-CCCAGAGATA-3’. Nestin-Cre genotyping was performed using the primers 5’-CTGGGCGCCGTTCTTGC-3’ and 5’-GGCTTCGCAGAAATTTC-3’.
Figure 1  PPARγ in myeloid cells modulates the effector phase of EAE. (A) LysM-Cre mice were crossed to R26-eYFP<sup>fl/wt</sup> reporter mice to analyse successful recombination i.e. YFP expression, by flow cytometry in different immune cell populations. Graph shows the recombination efficiency in the named immune cell populations of <i>n</i> = 3 cre<sup>+</sup> mice. See Supplementary Fig. 1A for corresponding histograms. Numbers indicate mean percentage of YFP<sup>+</sup> cells. (B) MOG<sub>35–55</sub>-EAE was induced by active immunization of LysM-PPAR<sub>γ</sub><sup>WT</sup> (<i>n</i> = 12) or LysM-PPAR<sub>γ</sub><sup>KO</sup> (<i>n</i> = 13) mice, and mice were clinically monitored. The mean clinical score ± SEM of one representative experiment out of five is shown. (C and D) Histopathological profile (left) and quantification (right) of mice at Day 35 after immunization. (C) Demyelination [luxol fast blue (LFB)] and axonal damage [amyloid precursor protein (APP)] (<i>n</i> = 5 per group); (D) Quantification of immune cell infiltration (MAC-3 for macrophages/microglia, CD3 for T cells) (<i>n</i> = 5). Scale bars indicate 500 μm in the overview and 50 or 200 μm in the magnified detail. Graphs depict mean results ± SEM. (E and F) Expression of (E) inflammatory mediators and (F) chemokines in the CNS of EAE-diseased LysM-PPAR<sub>γ</sub><sup>WT</sup> and LysM-PPAR<sub>γ</sub><sup>KO</sup> mice was determined by quantitative real-time reverse transcriptase polymerase chain reaction on Day 21 (<i>n</i> = 10 per group). Data were normalized to endogenous GAPDH and are displayed as n-fold induction compared with healthy LysM-PPAR<sub>γ</sub><sup>WT</sup> or LysM-PPAR<sub>γ</sub><sup>KO</sup> mice, respectively. Data are expressed as mean ± SEM. (G) MOG-EAE was induced in Nestin-PPAR<sub>γ</sub><sup>WT</sup> (<i>n</i> = 6) and Nestin-PPAR<sub>γ</sub><sup>KO</sup> mice (<i>n</i> = 6) and disease course was monitored. The mean clinical score ± SEM of one representative experiment out of four is shown. *<i>P</i> < 0.05; **<i>P</i> < 0.01; ***<i>P</i> < 0.001. iNOS = inducible nitric oxide.
Cell culture

For preparation of mixed glial cultures, the cerebrum from post-natal Days 1–5 LysM-PPARγKO and LysM-PPARγWT or C57BL/6 mice was stripped of meninges. The cortices were mechanically dissociated as described previously (Wang and Neumann, 2010), seeded into culture flasks in Dulbecco’s Modified Eagle’s Medium supplemented with 10% foetal calf serum, 50 μM β-mercaptoethanol, 2 mM l-glutamine, 1% non-essential amino acids, 105 U penicillin and 0.1 g/l streptomycin. After 2 weeks, microglial cells were harvested by vigorously tapping the flasks, the remaining astrocytes were used for experiments where indicated.

Primary neuronal cultures were prepared from hippocampi of C57BL/6 mice embryos (embryonic Day 15–16). Hippocampi were isolated, dispersed mechanically and seeded on 4-well chamber culture dishes, which were pretreated with 0.01 mg/ml poly-L-ornithine and 10 μg/ml laminin. The cells were cultured in basal medium Eagle supplemented with 2% B-27 supplement (Gibco-BRL, Invitrogen), 1% glucose (Sigma), and 1% foetal calf serum. Cells were cultured for 4 days to obtain morphologically mature neurons. Then, neurons were co-cultured with microglial cells.

Bone marrow-derived macrophages were generated as described previously (Raccoosin and Swanson, 1989). For PPARγ-activation in microglia or bone marrow-derived macrophages, 10 μM pioglitazone (Axxora) was added for 7 days prior to stimulation. Bone marrow-derived macrophages were differentiated into monocytes with 100 ng/ml lipopolysaccharide (LPS) (Axxora) was added for 7 days prior to stimulation. Bone marrow-derived macrophages were generated as described previously (Koch et al., 2011). Differentiation of induced pluripotent stem cells into long-term neuroepithelial stem cells was performed following an established protocol (Koch et al., 2009). For astroglial differentiation, human-induced pluripotent stem cell-derived long-term neuroepithelial stem cells were seeded at a density of 4.2 × 10⁴/cm² in long-term neuroepithelial stem cell proliferation medium [Dulbecco’s Modified Eagle’s Medium/F12 containing 1:100 N2-supplement (Invitrogen), 10 ng/ml basic fibroblast growth factor, 10 ng/ml epidermal growth factor (both R&D systems), 1:1000 B27-supplement (Invitrogen)] on dishes coated with Matrigel™ (BD Biosciences). Medium was changed the next day to glial differentiation medium [Dulbecco’s Modified Eagle’s Medium/F12 containing 1:100 N2-supplement, 10% foetal calf serum (Gibco), 30 ng/ml CTNF (R&D systems), 45 nM triiodothyronine (Sigma)]. Medium was changed every other day. Cells were trypsinized at 90% confluency (usually Days 5–6 of differentiation) and replated on gelatine-coated dishes at a ratio of 1:2 in glial proliferation medium (Dulbecco’s Modified Eagle’s Medium/F12 containing 1:100 N2-supplement, 20 ng/ml basic fibroblast growth factor, 20 ng/ml epidermal growth factor, 45 nM triiodothyronine). After two additional passages glial proliferation medium was supplemented with 10 μM forskolin for further cultivation.

Astrocyte co-cultures

For all co-culture experiments, murine/human astrocytes were co-cultured with murine bone marrow-derived macrophages or human monocytes at a ratio of 1:2 and CCL2 release into the supernatant was determined by ELISA after 24 h. Before set-up of co-cultures, PPARγWT or PPARγKO bone marrow-derived macrophages were pre-stimulated with LPS and IFNγ for 24 h, thoroughly washed, and subsequently cultured with primary astrocytes. Alternatively, pioglitazone-treated PPARγWT, PPARγKO and PPARγKO bone marrow-derived macrophages were co-cultured with pre-activated OT-II T cells in the presence of 5 μg/ml OVA323–335 (antigen-specific) or 20 μg/ml MOG35–55 (unrelated antigen). After 24 h, bone marrow-derived macrophages were isolated by immunomagnetic bead separation using CD11b+ beads according to the manufacturer’s protocol (Miltenyi Biotec) and co-cultured with primary astrocytes. For human co-culture, monocytes were prestimulated with 1 μg/ml LPS and 1000 U/ml IFNγ for 48 h.

Induction of experimental autoimmune encephalomyelitis

For active immunization, EAE was induced as described previously (Klotz et al., 2009). For adoptive transfer EAE, mice were immunized with 50 μg MOG35–55 peptide (Biotrend) emulsified in complete Freund’s adjuvant (CFA) subcutaneously in the flank. After 10 days, splenic cells were isolated and cultured for 72 h in the presence of 20 μg/ml MOG35–55 (Pineda) and 20 ng/ml IL-12 (R&D Systems). Cells (8 × 10⁶) were injected intraperitoneally into recipient mice, which also received 100 ng Bordetella pertussis toxin (Sigma) on Days 0 and 2 after adoptive transfer. Clinical assessment of EAE was performed daily using a scale ranging from 0 to 8: 0, no paralysis; 1, limp tail; 2, ataxia or unilateral hind limb paresis; 3, severe unilateral or weak bilateral hind limb paresis; 4, severe bilateral hind limb paresis; 5, complete bilateral hind limb plegia; 6, complete bilateral hind limb plegia and partial forelimb paresis; 7, severe tetraparesis/plegia; and 8, moribund/dead animals.

Oral treatment with pioglitazone

Mice were treated daily with 30 mg/kg body weight pioglitazone (Actos; Takeda Pharmaceuticals) suspended in 0.5% carboxymethyl-cellulose by oral gavage. Control mice received the vehicle only.
Histology
For immunohistological analysis, the CNS of EAE-diseased mice was perfused on Day 35 post immunization with Gey’s balanced salt solution and heparin (Ratiopharm) and afterwards fixed in 4% paraformaldehyde. The spinal cord was isolated and immunohistochemistry was performed as described previously (Prinz et al., 2006, 2008).

Depletion of CCR2+ inflammatory monocytes
CCR2+ inflammatory monocytes were depleted in LysM-PPARγKO or LysM-PPARγWT mice during EAE by intraperitoneal injection of 20 μg αCCR2 (MC-21) or the isotype control Ig (RatIgG2b; BD Biosciences) every 24 h from Days 13 to 17 after induction of EAE as described (Bruhl et al., 2007; Mildner et al., 2009). Specific depletion of inflammatory monocytes was monitored in the blood 8 h after the first injection.

In-vivo monocyte differentiation experiments
For in vivo labelling of circulating Ly-6Chigh inflammatory monocytes during EAE, mice were treated as described before (Getts et al., 2008). Briefly, on Day 12 after MOG-immunization, mice received 200 μl clodronate liposomes intravenously and 12 h later, 0.5 μm FITC-labelled Fluoresbrite® polystyrene beads (Polyscience) were injected intravenously, which resulted in stable labelling of Ly-6Chigh monocytes. In contrast, Ly-6Clo monocytes were preferentially labelled in phosphate-buffered saline liposome-treated mice. Analysis of CNS-invading monocytes was performed 12 h after FITC-bead labelling.

On Day 13 after EAE immunization, 1 × 10^7 Ly-6ChighCD115+ monocytes, isolated from the bone marrow of C57BL/6 (CD45.2) mice by immunomagnetic separation, were adoptively transferred into C57BL/6 mice carrying the congenic marker CD45.1. Twelve hours later, flow cytometrical analysis of CNS-invading CD45.2+ monocytes was performed. Transferred monocytes were discriminated from endogenous monocytes by surface marker staining with antibodies for CD45.1 and CD45.2, respectively.

Transfer of LysM-PPARγWT and LysM-PPARγKO monocytes
MOG-EAE was induced in wild-type mice. On Days 12, 14, 16 and 19 of EAE, CD115+ monocytes were isolated by immunomagnetic separation from the bone marrow of 15 healthy LysM-PPARγWT and 15 healthy LysM-PPARγKO mice and 1 × 10^7 Ly-6ChighCD115+ monocytes per mouse and per time point were transferred. The clinical score was monitored daily.

Isolation of central nervous system mononuclear cells
For mononuclear cell isolation, mice were anaesthetized with Avertin (Sigma), perfused with Gey’s balanced salt solution and heparin, and brains and spinal cords were removed carefully. Tissue was mechanically dissected and digested with 1 mg/ml collagenase A (Sigma) in phosphate-buffered saline for 30 min at 37°C while shaking. The tissue was homogenized, washed and resuspended in 30% isotonic Percoll®, which was underlayered with 37 and 70% Percoll®. After gradient centrifugation, mononuclear cells were collected from the 30:37% and the 37:70% interface, washed extensively with phosphate-buffered saline, and analysed by flow cytometry.

Detection of nitric oxide, TNFα, IL-6 and CCL2
To evaluate nitric oxide production, nitrate concentrations were measured in the supernatants of cells after 48 h using the Griess Reagent (Invitrogen) according to the manufacturer’s protocol. Release of IL-6, TNFα and CCL2 in supernatants was quantified by ELISA after 24 and 48 h, respectively, according to the manufacturer’s recommendation.

Determination of microglial neurotoxicity
Pioglitazone-treated or untreated primary microglia were harvested and stimulated with 1 μg/ml LPS + 10 ng/ml IFNγ. After 48 h, microglia were resuspended in neuronal medium without LPS and IFNγ and added to primary neurons at a ratio of 1:5 (microglia:neurons). After 24 h, immunohistological staining of neurons and analysis of relative neuronal neurite density was performed as previously described (Wang and Neumann, 2010).

Flow cytometry
Surface marker staining was performed as described previously, using a BD Canto II (BD Biosciences) (Burgdorf et al., 2007). For intracellular IFNγ and IL-17A staining, T cells were isolated from the CNS of EAE mice and restimulated ex vivo for 4 h with 5 ng/ml PMA and 200 ng/ml ionomycin in the presence of monensin and brefeldin A (eBioscience). Staining was performed using the BD Cytofix/ Cytoperm™ Fixation/Permeabilization Solution Kit (BD Bioscience) according to the manufacturer’s protocol. Results were analysed with FlowJo software (Tree Star).

Antibodies
Fluorochrome-labelled antibodies against murine CD3, CD4, CD8α, CD11b, CD11c, CD40, CD45, CD45.1, CD45.2, F4/80, MHC-II, Ly-6C, Ly-6G, IL-17A, TNFα and IFNγ for flow-cytometric analysis were obtained from eBioscience and BD Bioscience. Antibodies against human CD40, CD54, CD80, CD86 and HLA-DR were purchased from Biolegend and Miltenyi Biotec. Antibodies for ELISA (murine and human, respectively, TNFα, IL-6 and CCL2) were obtained from Ebioscience and R&D Systems.

Quantitative real-time reverse transcriptase polymerase chain reaction
Isolation of messenger RNA from microglia and bone marrow-derived macrophage was performed with RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Tissue from the upper cervical myelon was isolated on Day 21 after immunization and messenger RNA was isolated with Trizol Reagent (Invitrogen) according to the manufacturer’s protocol. Messenger RNA (1 mg) was transcribed into complementary DNA by reverse transcriptase with Superscript III (Invitrogen) according to the manufacturer’s protocol. Tissue from the upper cervical myelon was isolated on Day 21 after immunization and messenger RNA was isolated with Trizol Reagent (Invitrogen) according to the manufacturer’s protocol. Messenger RNA (1 mg) was transcribed into complementary DNA by reverse transcriptase with Superscript III (Invitrogen) according to the manufacturer’s protocol. Expression of messenger RNA was quantified in triplicates by quantitative real-time reverse transcriptase polymerase chain reaction using gene-specific primers (IL-1β, IL-12 p40, TNFα, IFNγ, FIZZ, Ym1/2; Supplementary Table 1),
QuantiTect Primer Assays [iNOS, murine Arginase 1 (Arg-1)] (Qiagen), or FAM-labelled TaqMan probes (human Arginase-1, CCL2, CCL3, CCL5, CXCL10, GAPDH; Applied Biosystems), or Vic-labelled TaqMan^® probes (18 s). The polymerase chain reaction mixture was prepared with Power SYBR^® Green PCR Master Mix or TaqMan^® Gene Expression Master Mix (Applied Biosystems) according to the manufacturer's protocol. Amplification of complementary DNA was carried out on an ABI Prism 7900 HT cycler. Data quantification was performed by the ΔΔCt method and normalized to GAPDH expression as described in the User Bulletin 2 of the ABI Prism manufacturer.

**PPARγ-ablation in cells**

For detection of PPARγ-ablation in various cells, messenger RNA was isolated and reverse-transcribed into complementary DNA as described (see above). Reverse transcriptase polymerase chain reaction was performed using primers: 5'-TATCAGGATCTCCGACACGC-3' and 5'-GTACGGTTCGACAG-CAGTGTAGCAC-3' for PPARgamma. Alternatively, LysMCre mice were crossed to R26-eYFP^fl/wt^ mice. Successful recombination, i.e. yellow fluorescent protein (YFP) expression, was analysed by flow cytometry in granulocytes (SSC^hi CD1b^+^), splenic macrophages (CD11b^+^ F4/80^+^), blood-derived resident monocytes (SSC^lo CD11b^+^ Ly-6Chi), CNS-derived adult microglia (CD11b^+^), CNS-resident cells contributes to modulation of local immune responses within the CNS during the effector phase of EAE.

**Statistics**

All data are expressed as mean ± SEM. Statistical analysis was performed using unpaired Student’s t-test for all experiments except for EAE experiments, where we used the ANOVA plus Bonferroni test; P < 0.05 were considered significant; P < 0.01 were considered highly significant.

**Results**

**Cell-intrinsic function of PPARγ in myeloid cells during the effector phase of experimental autoimmune encephalomyelitis**

Previously, we have shown that PPARγ in CD4^+^ T cells controls development of T_{h}17 differentiation and T_{h}17-dependent induction of CNS autoimmunity (Klotz et al., 2007). The effect of PPARγ-ablation in T cells caused earlier disease onset but not aggravation of disease during the effector phase, which suggested that autoreactive CD4^+^ T cells mainly contributed to disease initiation but were not the critical mediators of local autoimmune damage. Therefore, we wondered whether other immune cells were critically involved in disease progression. To address this question, we generated myeloid cell-specific PPARγ knockout mice by crossing LysM-Cre mice with transgenic mice carrying loxP sites within the PPARγ gene (LysM-PPARγ^KO^). In order to quantify recombination efficiency, we crossed LysM-Cre mice with R26-eYFP^R26^ reporter mice and assessed YFP expression in immune cells by flow cytometry. As shown in Fig. 1A and Supplementary Fig. 1, LysM-Cre mice exhibited high recombination-efficiency in monocytes, macrophages and granulocytes, and lower recombination efficiency in CNS-ex vivo-derived adult microglia, whereas dendritic cells, T cells, and B cells were not affected [Supplementary Fig. 1 and Clausen et al. (1999)]. Upon EAE induction by immunization with MOG35-55, LysM-PPARγ^KO^ mice compared with wild-type mice showed a similar development of disease severity during the T cell-mediated induction phase (Fig. 1B). Instead, LysM-PPARγ^KO^ mice exhibited a significantly aggravated disease course during the effector phase beginning at Day 15 (Fig. 1B) and histopathological analysis of the spinal cords from LysM-PPARγ^KO^ mice during the effector phase at Day 35 after disease induction revealed exacerbated demyelination and axonal damage (Fig. 1C). At this time point, CNS infiltration by macrophages and T cells was significantly increased (Fig. 1D). Similar to findings in inflammatory brain lesions in patients with multiple sclerosis (Bruck et al., 1995; Barnett et al., 2006), we found a clear predominance of macrophages over T cells in LysM-PPARγ^KO^ mice (Fig. 1D). Augmented disease severity during the effector phase in LysM-PPARγ^KO^ mice was mirrored by an increased inflammatory milieu within the CNS characterized by enhanced expression of pro-inflammatory mediators and chemokines involved in immune cell recruitment (Fig. 1E and F). Hence, in the absence of the cell-intrinsic regulatory function of PPARγ, myeloid cells facilitate immune cell recruitment and generation of a pro-inflammatory milieu, which contributes to enhanced demyelination and neuronal damage. As expected, pharmacological PPARγ activation by the synthetic agonist pioglitazone not only ameliorated the disease course (Nino et al., 2001; Klotz et al., 2009) but also attenuated local pro-inflammatory responses and eventually CNS damage (Supplementary Fig. 3). To exclude a role of PPARγ in other CNS-resident cells during EAE, i.e. neurons, astrocytes and oligodendrocytes, we generated mice that lack PPARγ in cells derived from neuronal and glial precursors [Nestin-PPARγ^KO^ mice; Supplementary Fig. 3 and Tronche et al. (1999)]. Importantly, ablation of PPARγ in those CNS-resident cells did not influence the disease course of EAE at all (Fig. 1G). Taken together, PPARγ in myeloid cells but not in other CNS-resident cells contributes to modulation of local immune responses within the CNS during the effector phase of EAE.

**Systemic and local T cell responses are not affected in LysM-PPARγ^KO^ mice**

As myeloid cells function as antigen-presenting cells to prime T cells and initiate (auto)immunity, we investigated whether myeloid cell-specific PPARγ-ablation increases naïve T cell priming thereby contributing to aggravated CNS inflammation. Characterization of MOG-specific T cell responses in LysM-PPARγ^KO^ mice at different time points after immunization revealed no increase in the frequency of IFNγ- or IL-17A-producing CD4^+^ T cells in the draining lymph nodes of LysM-PPARγ^KO^ mice at Day 7 and Day 14 during disease initiation (Fig. 2A) or within the CNS at Day 14 and Day 27 (Fig. 2B). Moreover, CD8^+^ T cell responses were also neither altered at Day 27 in the CNS of LysM-PPARγ^KO^ mice (Fig. 2C) nor at earlier time points in the periphery (data not shown). Thus, lack of PPARγ in LysM^ko^ myeloid cells does not trigger augmented MOG-specific T cell responses. Additionally, we adoptively
transferred activated MOG-specific wild-type T cells into LysM-
Pparg/C13 KO mice or wild-type littermates. Again, LysM-Pparg/C13 KO mice developed an aggravated disease course during the effector phase of EAE when compared with wild-type controls (Fig. 2D), which indicates that differences in T cell priming do not substantially contribute to the observed aggravation during the effector phase of EAE in LysM-Pparg/C13 KO mice. Albeit the difference was somewhat smaller than after active MOG immunization (Fig. 1A)—probably due to increased overall disease severity in this particular model—it was consistently observed in independent experiments incorporating a total of 27 LysM-Pparg/C13 KO mice and 22 LysM-Pparg/C13 WT mice. Together, these data indicate that Pparg/C13-ablation in myeloid cells did not augment T cell responses but rather aggravated local inflammation within the CNS.

PPARGγ-ablation results in an enhanced activation status of myeloid cells during experimental autoimmune encephalomyelitis

To further characterize the role of Ppargγ in myeloid cells during the effector phase of EAE we quantified the numbers of CNS-infiltrating macrophages (CD45hiLy-6Chi) and resident microglial cells (CD45lowLy-6Clow) and observed a 2-fold increase in total cell numbers on Day 21 of EAE in LysM-Ppargγ/C13 KO mice compared with littermate control mice (Fig. 3A and B), consistent with results shown in Fig. 1C. We next investigated whether Ppargγ also influenced the activation status of these cells during EAE and determined expression of CD40 and MHC-II, molecules critical for interaction with autoreactive T cells and therefore involved in disease deterioration (Becher et al., 2001; Greter et al., 2005). CNS-infiltrating macrophages and microglia in LysM-Ppargγ/C13 KO mice showed increased expression of CD40 (Fig. 3C) and MHC-II (Fig. 3D). Thus, Ppargγ ablation in myeloid cells increases not only local myeloid cell numbers in the CNS but also enhances their activation status, which points to a so far unrecognized role of CNS-located myeloid cells during the effector phase of EAE.

PPARGγ controls T cell-mediated licensing of macrophages

We hypothesized that during EAE, myeloid cells in the CNS were activated locally by autoreactive T cells and that ablation of Ppargγ facilitated this activation step. This prompted us to test whether antigen-specific interaction with MOG-specific CD4+ T cells results in myeloid cell activation. Similar to the observation...
in vivo, we observed increased production of nitric oxide and TNFα upon antigen-specific interaction of PPARγKO compared with PPARγWT bone marrow-derived macrophages with autoreactive MOG-specific effector 2D2 T cells (Fig. 4A). Of note, no production of these neurotoxic mediators was observed when T cells did not see their cognate antigen (Fig. 4A). On the other hand, pharmacological activation of PPARγ in macrophages by the synthetic ligand pioglitazone suppressed production of proinflammatory mediators elicited by antigen-specific interaction with CD4+ T cells (Fig. 4B). Importantly, antigen-specific activation of macrophages was not restricted to 2D2 T cells, but was also observed when macrophages were co-cultured with OVA-specific effector OT-II T cells, which was similarly found to be modulated by PPARγ activity in macrophages (Fig. 4C). In addition to induction of proinflammatory mediators, antigen-specific interaction with either MOG- or OVA-specific CD4+ T cells elicited upregulation of the co-stimulatory molecule CD40 on macrophages (Fig. 4D). This upregulation was also controlled by cell-intrinsic PPARγ-activity, as it was enhanced in PPARγKO macrophages and reduced in PPARγ-activated macrophages (Fig. 4D). Together, these results indicate that encephalitogenic CD4+ T cells license myeloid cells upon antigen-specific interaction, which results in induced expression of effector molecules that drive CNS inflammation and organ damage (Hendriks et al., 2005). Importantly, PPARγ in macrophages controls this licensing step.

Based on these findings we next asked whether pharmacological PPARγ-activation in vivo would also influence the functional outcome of myeloid cell cross-talk with disease-triggering T cells during the effector phase of EAE (Day 21). CNS macrophages and microglia of pioglitazone-treated mice exhibited reduced expression of CD40 and MHC-II compared with vehicle-treated mice (Fig. 4E). Importantly, this reduction in CD40 and MHC-II expression on CNS-infiltrating macrophages by pioglitazone treatment was mediated by PPARγ-activation directly in myeloid cells, because we did not observe such reduction in pioglitazone-treated LysM-PPARγKO mice (Fig. 4F). Hence, these data indicate that in vivo, T cell-mediated licensing of myeloid cells is controlled by myeloid cell-intrinsic PPARγ.

PPARγ-mediated control of myeloid cell activity regulates recruitment of CCR2+ inflammatory monocytes that drive the effector phase of experimental autoimmune encephalomyelitis

Recently, CCR2+ inflammatory monocytes have been identified as the subpopulation of myeloid cells that crucially contributes to EAE pathogenesis during the effector phase (Geissmann et al., 2003; King et al., 2009; Mildner et al., 2009). It has been demonstrated...
Figure 4 PPARγ controls T cell-mediated licensing of myeloid cells and regulates their activation status during EAE. (A and B) Bone marrow-derived macrophages were co-cultured with activated 2D2 T cells at a ratio of 2:1 (BM-M:T) in the presence of their specific antigen MOG35–55 (5, 10 or 20 μg/ml) or unrelated antigen OVA223–339 (5, 10 or 20 μg/ml). Nitric oxide (NO) or TNFα release into the supernatant was determined by Griess assay after 48 h, and ELISA after 24 h, respectively. (A) Co-culture of PPARγWT or PPARγKO bone marrow-derived macrophages with activated 2D2 T cells. (B) Bone marrow-derived macrophages were pretreated with 10 μM pioglitazone (PIO) for 7 days or left untreated (w/o PIO), and co-cultured with activated 2D2 T cells. (C) Pioglitazone-treated PPARγWT, untreated PPARγWT or PPARγKO bone marrow-derived macrophages were co-cultured with pre-activated OT-II T cells in the presence of the specific antigen OVA (5 μg/ml) or the unrelated antigen MOG (5 μg/ml), and nitric oxide and TNFα was determined as in (A). (D) CD40 expression on CD11b+ cells was determined after 24 h by flow cytometry on bone marrow-derived macrophages co-cultured with activated 2D2 T cells or OT-II T cells in the presence of the cognate antigen or an unrelated antigen as in A–C, respectively. (E) Thirty milligram per kilogram body weight pioglitazone or vehicle only (carboxymethylcellulose, CMC) was administered daily by oral gavage to C57BL/6 wild-type mice and on Day 7 EAE was induced. On Day 21 after EAE-immunization, CD40 and MHC-II expression on CNS-macrophages and microglia was analysed by flow cytometry as described in Fig. 3 (n = 5 per group). (F) LysM-PPARγKO mice were orally treated with pioglitazone or vehicle only as described in (E), macrophages were isolated from the CNS during the effector phase (Day 21) and flow cytometrically analysed for CD40 and MHC-II expression (n = 4 per group). Numbers in histograms express mean MFI ± SEM; accompanying graphs depict mean percentage ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.
that during EAE the chemokine CCL2 is responsible for CCR2+ immune cell recruitment (Huang et al., 2001). Interestingly, we observed that PPARγ in myeloid cells regulated local CCL2 expression within the CNS during EAE, as LysM-PPARγKO EAE mice exhibited significantly enhanced CCL2-messenger RNA levels within the inflamed CNS, whereas PPARγ activation during EAE resulted in reduced CCL2 expression levels within the CNS (Fig. 5A). During CNS inflammation in multiple sclerosis and EAE, astrocytes have been shown to be the main source of CCL2 both in mice and humans (Van Der Voorn et al., 1999; Giraud et al., 2010). We therefore wondered whether activated CNS myeloid cells might modulate astrocytic CCL2 production. In a first step, we exposed primary murine astrocytes to pre-activated PPARγWT or PPARγKO bone marrow-derived macrophages, and subsequently assessed CCL2 production. Activated macrophages themselves produced only low amounts of CCL2 (Fig. 5B), but elicited substantial CCL2 production in co-cultured astrocytes (Fig. 5B), whereas resting macrophages did not induce astrocytic CCL2 production (Fig. 5B). Importantly, lack of PPARγ in myeloid cells significantly augmented their capacity to elicit astrocytic CCL2 production (Fig. 5B). Following this line further, we asked whether antigen-specific interaction with activated CD4+ T cells equally enables macrophages to evoke astrocytic CCL2 production, as the interaction with autoreactive T cells can be considered as more disease-relevant stimulus compared with LPS-stimulation. Macrophages evoked substantial astrocytic CCL2 production selectively following antigen-specific interaction with CD4+ T cells (Fig. 5C). With regard to the experimental setup, it is important to note that following interaction with effector CD4+ T cells, macrophages were purified prior to astrocyte co-culture. Moreover, activated CD4+ T cells themselves did not evoke astrocytic CCL2 production upon co-culture (Fig. 5C). As observed before, lack of PPARγ in macrophages augmented, whereas pharmacological PPARγ activation reduced astrocytic CCL2 production (Fig. 5C). Of note, we observed no relevant CCL2 production by either resting or activated Tn1 or Tn17 cells (Supplementary Fig. 4A), suggesting that autoreactive T cells during EAE are not a source of CCL2. To exclude altered astrocyte function in the CNS of LysM-PPARγKO mice, we determined CCL2 production by astrocytes isolated from LysM-PPARγKO mice or their wild-type littermates and did not observe any differences (Supplementary Fig. 4B). Together, these data suggest that CNS myeloid cells become activated upon antigen-specific interaction with autoreactive T cells and then engage in further cross-talk with astrocytes triggering astrocytic CCL2-production; this cross-talk is modulated by PPARγ in myeloid cells.

Based on these results we next addressed the question whether PPARγ also influences the function of CCR2+ inflammatory monocytes during the effector phase of EAE. To address this experimentally, we depleted CCR2+ Ly-6Chi inflammatory monocytes...
Figure 5  PPARγ-mediated control of myeloid cell activity regulates CCR2⁺-dependent recruitment of inflammatory monocytes that drive the effector phase of EAE. (A) EAE was induced in LysM-PPARγ<sup>WT</sup> (n = 10) or LysM-PPARγ<sup>KO</sup> (n = 10) mice (top) or pioglitazone- or vehicle-treated mice (n = 10; bottom). Mean CCL2 messenger RNA expression ± SEM in the CNS was determined on Day 21 of EAE by quantitative real-time reverse transcriptase polymerase chain reaction. Data were normalized to endogenous GAPDH expression and are displayed as n-fold induction compared with healthy LysM-PPAR<sup>WT</sup> or LysM-PPAR<sup>KO</sup> mice (top) and healthy carboxymethylcellulose (CMC) or pioglitazone (PIO)-treated C57BL/6 mice (bottom), respectively. (B) PPARγ<sup>WT</sup> and PPARγ<sup>KO</sup> bone marrow-derived macrophages were stimulated with LPS and IFNγ for 24 h (stim) or bone marrow-derived macrophages were prestimulated with LPS and IFNγ for 1 h, thoroughly washed, and subsequently co-cultured with unstimulated primary murine astrocytes at a ratio of 1:1 for 24 h. CCL2 secretion was measured by ELISA. ***Statistical significance between wild-type and knockout bone marrow-derived macrophages; ###Statistical significance between unstimulated astrocytes and co-culture groups (P < 0.001). (C) Primary murine astrocytes were cultured either alone, with activated OT-II T cells, with unstimulated bone marrow-derived macrophages or with bone marrow-derived macrophages that had been pre-activated in co-culture with OT-II effector T cells in the presence of OVA<sub>323–339</sub> or MOG<sub>35–55</sub> for 24 h. After T cell interaction, bone marrow-derived macrophages were purified and subsequently co-cultured with astrocytes for 24 h (ratio 2:1). CCL2 secretion was determined by ELISA. *Statistical significance between astrocytes alone and indicated co-culture groups; †Statistical significance within one particular co-culture group. (D–F) CCR2⁺ inflammatory monocytes were depleted during EAE in LysM-PPARγ<sup>KO</sup>
by repetitive injection of the αCCR2 antibody MC-21 (Supplementary Fig. 5) (Bruhl et al., 2007; Mildner et al., 2009) and determined its effect on disease progression. Depletion of CCR2+ cells not only ameliorated the EAE disease course, but also abolished the observed differences in disease severity between LysM-PPARγKO mice and their wild-type littermates (Fig. 5D). Concomitantly, depletion resulted in reduced expression levels of pro-inflammatory mediators within the CNS and furthermore completely abrogated the differences in pro-inflammatory gene expression between LysM-PPARγKO and LysM-PPARγWT mice (Fig. 5E). Importantly, depletion of CCR2+ cells during EAE nearly completely abrogated local CCL2 expression in the CNS indicating the existence of a self-perpetuating recruitment of CCL2-responsive cells at later stages of disease (Fig. 5E). At the single cell level, PPARγKO macrophages from MC-21-treated animals still exhibited increased expression levels of MHCI and CD40 (Fig. 5F), suggesting that the antibody treatment did not alter the activation status of PPARγ-ablated CNS-resident myeloid cells per se.

To more specifically address the relevance of PPARγ in inflammatory monocytes during the effector phase of EAE we adoptively transferred PPARγKO or PPARγWT monocytes into MOG-immunized wild-type mice during the effector phase on Days 12, 14, 16 and 19 after immunization. Transfer of PPARγWT monocytes slightly enhanced disease severity (Supplementary Fig. 6). Recipients of PPARγKO monocytes, however, exhibited a more aggravated effector phase, further pointing towards a monocyte-intrinsic effect of PPARγ to control disease activity.

Taken together, these results allow two important conclusions: first, local activation of CNS myeloid cells evokes astrocytic CCL2 production, which is then maintained by a sustained influx of inflammatory monocytes in a self-perpetuating fashion. Second, PPARγ in myeloid cells controls both recruitment and activity of CCR2+ inflammatory monocytes as central driving force for progression of CNS autoimmunity during EAE.

Organ-protective role of PPARγ in central nervous system-resident myeloid cells

Besides inflammatory monocytes, microglia cells also critically determine the extent of local inflammation and hence disease severity during the effector phase of EAE (Heppner et al., 2005). Given the prominent function of inflammatory monocytes in our experiments we wondered whether these cells might acquire microglial features within the inflammatory CNS environment. Several reports demonstrated that under certain conditions, e.g. viral encephalitis, and blood–brain-barrier injury after irradiation (Getts et al., 2008; Mildner et al., 2008), CNS-invading inflammatory monocytes can acquire a microglial phenotype. To follow the fate of inflammatory Ly-6Ch+ monocytes during EAE in vivo, we labelled Ly-6Ch+ monocytes using an in vivo clodronate-liposome-based bead labelling method as described previously (Getts et al., 2008). To this end, we injected FITC-labelled polystrene beads 12 h after depletion of monocytes from the blood by clodronate liposomes. This resulted in preferential labelling of Ly-6Ch+ monocytes in clodronate-liposome-treated mice but not in phosphate-buffered saline-liposome-treated mice (Supplementary Fig. 7). Already 12 h after labelling, we observed a distinct population of FITC-labelled inflammatory monocytes in the CNS of clodronate-liposome-treated EAE mice, demonstrating rapid recruitment during ongoing autoimmune inflammation (Fig. 6A and B). This experiment excluded extravasation of FITC-labelled beads into the inflamed CNS and subsequent uptake by resident microglial cells, because only very few labelled cells were found in the CNS of EAE mice that received phosphate-buffered saline-liposomes (Fig. 6A). We subsequently analysed CD45 expression levels on FITC+ cells, as CD45 is a reliable marker to distinguish macrophages from microglial cells (Getts et al., 2008), and found that within the short time frame of 12 h investigated here, nearly 10% of FITC-labelled inflammatory monocytes in the CNS already exhibited a microglial phenotype (Fig. 6A and B). We corroborated these results by an independent method, i.e. by transfer of CD45hi CD45.2+ monocytes into EAE-diseased CD45.1+ hosts (Fig. 6C). Again, we observed that ~5% of all transferred CD45.2+ monocytes now expressed intermediate levels of CD45 12 h after transfer, thus exhibiting a microglial phenotype.

Based on our results showing a critical role of PPARγ in the control of myeloid cell-mediated aggravation of EAE during the effector phase, we next investigated whether PPARγ also influences the organ damaging function of myeloid cells. Stimulation of macrophages and microglia resulted in production of TNFα and nitric oxide (Fig. 6D and E), molecules that mediate neurotoxicity (Hendriks et al., 2005). Indeed, pharmacological PPARγ activation suppressed release of these mediators and likewise PPARγ-ablated cells showed increased production of these molecules (Fig. 6D and E). Consistent with a regulatory role of PPARγ we found that in vivo PPARγ activation induced an immune-regulatory phenotype in monocytes (Supplementary Fig. 8A) that is also referred to as alternative or M2 activation (Fairweather and Cihakova, 2009). Of note, a similar induction of an M2 phenotype could be achieved by PPARγ activation in human monocytes (Supplementary Fig. 8B).

Figure 5 Continued

or LysM-PPARγWT mice by five injections (arrows indicate time points) of αCCR2 (MC-21; n = 20 per group) or isotype control (n = 15 per group). (D) Mean clinical score of two independent experiments ± SEM is shown. (E) Expression of inflammatory mediators and chemokines in the CNS was determined at Day 21 as in Fig. 1E and F (n = 5 per group, MC-21; n = 10 per group, isotype control Ig). Data are displayed as mean ± SEM. (F) MHCI and CD40 expression on CNS-macrophages at Day 21 after immunization (n = 5 MC-21; n = 3, isotype control); shown is one representative histogram overlay. *P < 0.05; **P < 0.01; ***P < 0.001. iNOS = inducible nitric oxide.
Figure 6  Organ-protective role of PPARγ in CNS-resident myeloid cells. (A and B) MOG–EAE was induced in C57BL/6 mice. On Day 12 post immunization, Ly-6C\textsuperscript{hi} inflammatory monocytes were FITC-labelled as described in methods. Twelve hours later, percentages of macrophages and microglia were analysed based on their CD45 expression by flow cytometry. (A) Gating on FITC\textsuperscript{+} CD11b\textsuperscript{+} Ly-6G\textsuperscript{−} CNS resident macrophages and microglia. (B) Mean percentage ± SEM of CD45\textsuperscript{hi} or CD45\textsuperscript{int} expressing FITC\textsuperscript{+} cells is shown (n = 5). (C) 1 x 10\textsuperscript{7} CD45.2\textsuperscript{+} CD115\textsuperscript{+} monocytes were transferred into EAE-diseased CD45.1\textsuperscript{+} mice on Day 12 post immunization and after 12 h, CNS-infiltrating CD45.2\textsuperscript{+} CD11b\textsuperscript{+} Ly-6G\textsuperscript{−} monocytes were analysed for CD45 expression (n = 5). (D and E) Production of neurotoxic mediators by bone marrow-derived macrophages or microglia after stimulation with LPS and IFN\gamma. Nitric oxide was determined by Griess reaction and TNF-α by ELISA (bone marrow-derived macrophages) or by flow cytometry (microglia) (n = 3 per group; one representative experiment out of at least four is shown). (D) Wild-type bone marrow-derived macrophages (BM-M) or microglia were treated with 10 \textmu M pioglitazone (PIO) or left untreated for 7 days prior to stimulation. (E) Nitric oxide (NO) and TNF-α detection in PPARγ\textsuperscript{WT} and (continued)
Importantly, primary microglial cells also showed PPARγ-mediated control of expression production of neurotoxic mediators (Fig. 6D and E), which had neuroprotective effects as assessed in a 24 h co-culture assay with primary neurons. PPARγ activation in microglial cells preserved neurite density under inflammatory as well as non-inflammatory conditions, indicating a continuous role for PPARγ-mediated control of microglial neurotoxicity (Fig. 6F).

**PPARγ in human monocytes dampens pro-inflammatory responses and restricts induction of astrocytic CCL2 production**

We next questioned whether human monocytes would also be responsive to PPARγ activation. In a first approach we analysed the inflammatory response of THP-1 cells, a well-characterized human monocytic cell line, following pharmacological PPARγ activation with rosiglitazone. We observed that upon LPS stimulation, rosiglitazone-treated THP-1 cells expressed significantly reduced levels of CD40, CD80 and HLA-DR (Fig. 7A) and produced significantly decreased amounts of TNFα (Fig. 7B). This prompted us to investigate whether PPARγ activation in primary human monocytes differentiated from peripheral blood mononuclear cells of healthy donors would also result in dampened inflammatory responses upon stimulation. Indeed, we observed that PPARγ activation resulted in significantly impaired upregulation of the activation markers CD40, CD54, CD80, CD86 and HLA-DR (Fig. 7C) as well as decreased production of the pro-inflammatory cytokines TNFα and IL-6 upon pro-inflammatory stimulation (Fig 7D). With regard to the development of novel therapies for patients with multiple sclerosis, we next asked whether monocytes from patients with multiple sclerosis were also responsive towards PPARγ-mediated control of proinflammatory activation. Importantly, rosiglitazone-treated monocytes from patients with multiple sclerosis also exhibited significantly reduced upregulation of both activation markers (Fig. 7E) and proinflammatory cytokines (Fig. 7F), following inflammatory stimulation. Additionally, we observed that human monocytes can be stimulated by pre-activated human Jurkat T cells, which was assessed by production of TNFα and IL-6 (Supplementary Fig. 9), and PPARγ activation in human monocytes by rosiglitazone limited such activation.

Since we observed that activated murine macrophages elicit CCL2 production in astrocytes (Fig. 5B and C) and that this CCL2 induction was limited upon PPARγ activation in macrophages, we next questioned whether activated human monocytes were also able to induce CCL2 production by human astrocytes. To this end, we set up a co-culture system of primary human monocytes and human-induced pluripotent stem cell-derived astrocytes (Supplementary Fig. 10). Also in the human system, preactivated monocytes both from healthy donors and patients with multiple sclerosis elicited substantial CCL2 production by astrocytes (Fig. 7G). Importantly, PPARγ activation in human monocytes significantly restricted this induction of astrocytic CCL2 production (Fig. 7G). These results could be corroborated using a human astrocytic cell line (U251 MG; Supplementary Fig. 11).

Together, these data indicate that also in humans, PPARγ modulates the activation status of myeloid cells from both patients with multiple sclerosis and controls, and that the interaction between human astrocytes and activated myeloid cells can elicit substantial astrocytic CCL2 production.

In summary, our data reveal a crucial role of PPARγ in myeloid cells and in inflammatory monocytes as key players during the effector phase of EAE. PPARγ in myeloid cells controls the outcome of T cell-induced cross-talk among CNS resident cells, which also dampens their innate effector functions resulting in reduced neuro-inflammation and amelioration of inflammation-induced neurotoxicity (Fig. 8).

**Discussion**

It is generally believed that CNS autoimmunity is a process promoted by autoreactive T cells (Stinissen et al., 1998). However, within inflammatory multiple sclerosis lesions or in the CNS of EAE mice, myeloid cells by far outnumber T cells and contribute to disease progression (Bruck et al., 1995; Barnett et al., 2006; Mildner et al., 2009). Yet it is not clear why CNS myeloid cells contribute to an autoimmune disease process that occurs in the absence of innate inflammatory stimulation. In this study, we made use of a cell-type specific ablation of the anti-inflammatory transcription factor PPARγ to study the contribution of myeloid cells to disease progression after encephalitogenic T cell invasion in CNS autoimmunity. These experiments revealed a hitherto unrecognized role of myeloid cells in driving progression of T cell-initiated CNS autoimmunity characterized by the following sequence of events: within the CNS, antigen-presenting myeloid cells were initially licensed by autoreactive T cells upon cognate antigen-recognition. The local inflammatory response elicited by this interaction during the early stages of CNS autoimmunity evokes CCL2 production through local cross-talk with astrocytes followed by recruitment of CCR2+ inflammatory monocytes into the CNS, which then function as the main effectors of organ damage. Furthermore we provide evidence that the nuclear receptor PPARγ in myeloid cells serves as a homeostatic control switch acting at several important steps of myeloid cell-driven neuro-inflammation.
Figure 7 PPARγ activation in human monocytes dampens pro-inflammatory responses and restricts induction of astrocytic CCL2 production. (A and B) Rosiglitazone (RSG)-treated or untreated THP-1 cells were analysed by flow cytometry (A) for expression of the indicated activation markers or (B) TNFα production after 24 h stimulation with LPS and IFNγ. Graphs depict the mean percentage of positive THP-1 cells ± SEM. One representative experiment out of three is shown. (C and D) Rosiglitazone-treated or untreated CD14+ primary monocytes from healthy donors were stimulated with LPS for 20 h. (C) Expression of the indicated activation markers by CD14+ cells was analysed by flow-cytometry. Histograms depict expression after LPS stimulation; graphs depict mean percentage (CD40, CD80, CD86, HLA-DR) or MFI (CD54) ± SEM. (D) TNFα and IL-6 release into the supernatant was determined by ELISA. Data from one representative healthy donor out of four is shown. (E and F) Analysis of rosiglitazone-treated or untreated monocytes from patients with multiple sclerosis was performed as in C and D. The results from one representative multiple sclerosis patient out of five are depicted.

(continued)
adoptive transfer of encephalitogenic wild-type CD4+ T cells still reveals a disease-promoting role of myeloid cells besides peripheral effector CD4+ T cells alone in the absence of additional innate autoimmunity can be induced by adoptive transfer of autoreactive et al Kierdorf in induction and aggravation of disease (Prinz et al., 2006; Kierdorf et al., 2010). However, this fails to explain how CNS autoimmunity can be induced by adoptive transfer of autoreactive effector CD4+ T cells alone in the absence of additional innate inflammatory activation. Here, we provide experimental evidence indicating that auto-antigen presentation by local myeloid cells in the CNS to infiltrating encephalitogenic T cells is crucial for myeloid cell activation and hence, propagation of CNS autoimmunity. This antigen-specific local cross-talk may represent a so far unrecognized turning point between T cell-driven induction and myeloid cell-driven effector phase of CNS autoimmunity. While our report is the first to describe antigen-specific but myeloid cell-mediated propagation of autoimmunity in the CNS, a similar key role for antigen-presenting cell licensing was made in autoimmune kidney disease. In a model of T cell-mediated kidney damage, initial cross-talk between CD4+ T cells and local antigen-presenting dendritic cells within the target organ was required for recruitment of autoreactive CD8+ T cells that then initiated progression of organ damage (Heymann et al., 2009). The importance of the initial antigen-specific cross-talk was demonstrated as dendritic cell depletion resolved established CD8+ T cell-dependent kidney pathology. Although in EAE it is not possible to selectively ablate CNS-resident myeloid cells, several arguments support our model: first, myeloid cell activation upon interaction with autoreactive T cells in vitro was strictly antigen-dependent. Secondly, adoptive transfer of encephalitogenic wild-type CD4+ T cells still caused disease aggravation during the effector phase in LysM-PPARγKO compared with LysM-PPARγWT mice, which reveals a disease-promoting role of myeloid cells besides peripheral T cell priming. It is important to note that in this system, transferred MOG-specific T cells were the only possible triggers of local myeloid cell activation. However, it is not possible to dissect whether encephalitogenic T cells are still required to sustain this process or alternatively, whether once initiated, myeloid cells function independently to drive immune cell recruitment and organ damage.

In the context of CNS autoimmunity, the identity of signals provided by T cells during this antigen-specific licensing step remains elusive, however, several molecules, e.g. CD40L, granulocyte-macrophage colony-stimulating factor and IFNγ have been identified as crucial mediators of T cell-mediated activation of antigen-presenting cells such as dendritic cells, macrophages and microglial cells (Koya et al., 2003; Koguchi et al., 2007; Min et al., 2010). Especially, CD40–CD40L interaction represents an interesting candidate in this context, as preformed CD40L exists in secretory lysosomes of effector CD4+ T cells and is quickly expressed in an antigen-specific fashion (Koguchi et al., 2007). In support of this hypothesis, we observed that the strength of human monocyte activation by human Jurkat T cells correlated to CD40L expression-levels on the T cell side (data not shown); however, it has to be pointed out that this setup did not involve antigen-specific interaction of monocytes and T cells. The general importance of CD40–CD40L interaction in CNS autoimmunity has convincingly been demonstrated (Becher et al., 2001; Chen et al., 2006), and microglial CD40 expression is essential for their full activation within the CNS, resulting in further effector T cell recruitment and expansion and hence disease progression (Ponomarev et al., 2006).

Myeloid cell-dependent propagation of autoimmune CNS disease comprised several key aspects: first, autoantigen-specific cross-talk with autoreactive T cells caused myeloid cell expression of molecules known to promote further T cell stimulation, such as CD40 and MHC class II. These molecules promote T cell-mediated CNS pathology (Becher et al., 2001) and it is tempting to...
speculate that they may also further accentuate myeloid cell activation in a self-perpetuating circle. Second, activated myeloid cells induce a further cross-talk chemokine expression, particularly CCL2, by astrocytes which is known to trigger recruitment of inflammatory CCR2+ monocytes into the CNS the key cell population that drives disease progression during the effector phase of EAE (Mildner et al., 2009). Both, during EAE and in acute inflammatory multiple sclerosis lesions, astrocytes seem to be the main source of CCL2, whereas myeloid cells and T cells do not produce much CCL2 under these conditions (Van Der Voorn et al., 1999; Giraud et al., 2010). Although we cannot formally rule out that myeloid cells or activated T cells contribute to local CCL2 production in vivo, we observed that astrocytic CCL2 production strongly exceeded CCL2 production by myeloid cells and CD4+ T cells in vitro. We now provide evidence for the first time that local interaction between activated myeloid cells and astrocytes is the key trigger for astrocytic CCL2 production. Importantly, this myeloid cell-induced astrocytic CCL2 production required pre-activation of myeloid cells e.g. by antigen-specific interaction with CD4+ effector T cells, which indicates that this licensing step facilitates cross-talk of myeloid cells with other cells involved in CNS autoimmunity. The exact mechanisms governing myeloid cell-induced expression of CCL2 by astrocytes remain unclear but it is important to note that astrocytic chemokine production was modulated by PPARγ activity in myeloid cells.

An important question raised by our results is whether endogenous PPARγ ligands might be relevant for continuous protection from inappropriate myeloid cell licensing under physiological conditions. This hypothesis is supported by the fact that cell-type specific ablation of PPARγ augmented disease progression, indicating that endogenous ligands were involved in PPARγ-mediated control of myeloid cell sensitivity. In other words, a particular baseline activity of PPARγ provided by local production of endogenous PPARγ ligands might serve as endogenous homeostasis signal preventing inappropriate immune activation and perhaps even contribute to the immune privileged state of the CNS. In support of this hypothesis, it has been observed that ablation of the enzyme involved in the production of several important PPARγ ligands, i.e. 12,15-lipoxygenase, resulted in enhanced CNS autoimmunity (Emerson and LeVine, 2004), whereas oral treatment with its substrate linoleic acid ameliorated clinical and histopathological signs of EAE (Harbige et al., 1995). In the CNS, IL-4 stimulation induces production of endogenous PPARγ ligands in microglial cells (Yang et al., 2002; Ponomarev et al., 2007) and high IL-4 levels are associated with resolution of CNS inflammation during EAE and in patients with multiple sclerosis (Falcone et al., 1998; Franciotta et al., 2000). IL-4 is also known to induce alternative activation of macrophages or M2 macrophages, and these M2 macrophages have a strong capacity to limit ongoing CNS autoimmunity (Butovsky et al., 2006; Weber et al., 2007). Interestingly, phenotype and function of PPARγ-activated myeloid cells during CNS autoimmunity are reminiscent of immunoregulatory macrophages. Following this line, we demonstrate that PPARγ activation in mice also induced alternative activation of circulating and splenic monocytes (Supplementary Fig. 8A), and likewise of human monocytes as previously shown (Bouhlel et al., 2007; Giraud et al., 2010). Reciprocal antigen-specific interaction between myeloid cells and T cells may establish a self-amplifying circle of mutual activation (2). Activated myeloid cells elicit local CCL2 production by astrocytes (3), which results in recruitment of CCR2+ inflammatory monocytes into the CNS (4). Newly recruited inflammatory monocytes perpetuate local CCL2 production and further immune cell recruitment and inflammation ultimately leading to aggravation of neurotoxicity and enhanced demyelination (5).

Figure 8 Cell-type specific ablation of PPARγ reveals a novel function of myeloid cells for driving progression of CNS autoimmunity during the effector phase of EAE. (A) Our results suggest a novel role of local myeloid cell—T cell interactions in the CNS during the effector phase of EAE where licensing of antigen-presenting myeloid cells by autoreactive CD4+ T cells initiates production of inflammatory and neurotoxic mediators (1). Reciprocal antigen-specific interaction between myeloid cells and T cells may establish a self-amplifying circle of mutual activation (2). Activated myeloid cells elicit local CCL2 production by astrocytes (3), which results in recruitment of CCR2+ inflammatory monocytes into the CNS (4). Newly recruited inflammatory monocytes perpetuate local CCL2 production and further immune cell recruitment and inflammation ultimately leading to aggravation of neurotoxicity and enhanced demyelination (5). (B) PPARγ in myeloid cells controls T cell-mediated licensing (1) and decreases the ability of myeloid cells to re-stimulate encephalitogenic T cells (2), thereby attenuating proinflammatory cytokine and chemokine production (3) and hence inflammatory monocyte recruitment (4), ultimately resulting in decreased neurotoxicity and demyelination (5).
Supplementary Fig. 8B). It is therefore an intriguing concept that the local microenvironment as a source of endogenous PPARγ-ligands may act as ‘anti-danger’ signal to influence the outcome of CNS autoimmunity by promoting alternative activation of myeloid cells.

T cell-specific PPARγ-ablation revealed a separate function of PPARγ in CNS autoimmunity characterized by enhanced differentiation of PPARγ<sup>C0</sup> T cells into T<sub>H</sub>17 cells that accelerate disease induction but do not affect the disease activity during the effector phase. The strategy of cell-specific PPARγ ablation allowed us to dissect the distinct and complementary roles of T cells and myeloid cells at the different disease stages. An important conclusion from our findings is that therapeutic approaches, which target myeloid cells, may be more effective for modulation of ongoing CNS autoimmunity in patients with multiple sclerosis than those targeting T cell responses. In patients with multiple sclerosis, we have previously shown that peripheral blood mononuclear cells exhibited decreased PPARγ expression levels, and that PPARγ activity inversely correlated with disease activity (Klotz et al., 2005). It is not possible to determine whether lower PPARγ expression levels are the consequence or cause of autoimmunity in these patients. However, in light of our findings presented here, these data suggest that in patients with multiple sclerosis, reduced PPARγ activity in myeloid cells may facilitate disease exacerbation due to an intrinsically lowered threshold towards T cell-mediated licensing. Importantly, we demonstrate here that PPARγ controls inflammatory activation of human monocytes and that this also holds true for monocytes from patients with multiple sclerosis. This indicates that myeloid cells from patients with multiple sclerosis can be targeted by PPARγ activation, which might in turn contribute to correction of decreased PPARγ activity observed in multiple sclerosis (Klotz et al., 2005). Even more interestingly, we provide evidence that primary human monocytes elicit CCL2 secretion by human astrocytes, which can be modulated by PPARγ in myeloid cells. This suggests that cross-talk between astrocytes and myeloid cells within the CNS, which results in CCL2-mediated immune cell recruitment, is also a feature of human CNS autoimmunity.

Taken together, our data suggest that myeloid cells amplify the disease-inducing potential of encephalitogenic T cells and perpetuate progression of CNS autoimmunity following initial antigen-specific interaction. We identify PPARγ in myeloid cells as critical factor in regulating such licensing and hence as a promising target for attenuating myeloid cell-mediated autoimmunity and ultimately organ damage.

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**Supplementary material**

Supplementary material is available at Brain online.

**References**


