Research Paper

Multiple Sclerosis

Multiple Sclerosis
0(00) 1–14

DOI: 10.1177/1352458510379243
msj.sagepub.com
SAGE

Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration

Joanna Mikita[1], Nadège Dubourdieu-Cassagno[1], Mathilde SA Deloire[1], Antoine Vekris[1], Marc Biran[2], Gérard Raffard[2], Bruno Brochet[1], Marie-Hélène Canron[1], Jean-Michel Franconi[2], Claudine Boiziau[1] and Klaus G Petry[1]

Abstract

Objectives: We investigated proinflammatory M1 and immunomodulatory M2 activation profiles of circulating monocytes in relapsing experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis, and tested whether altered M1/M2 equilibrium promotes CNS inflammation.

Results: Approaches of MRI macrophage tracking with USPIO nanoparticles and expression patterns of M1/M2 macrophages and microglia in brain and M1/M2 monocytes in blood samples at various disease stages revealed that M1/M2 equilibrium in blood and CNS favors mild EAE, while imbalance towards M1 promotes relapsing EAE. We consequently investigated whether M2 activated monocyte restoration in peripheral blood could cure acute clinical EAE disease. Administration of *ex vivo* activated M2 monocytes both suppressed ongoing severe EAE and increased immunomodulatory expression pattern in lesions, confirming their role in the induction of recovery.

Conclusion: We conclude that imbalance of monocyte activation profiles and impaired M2 expression, are key factors in development of relapses. Our study opens new perspectives for therapeutic applications in MS.

Keywords

cell transfer, cell activation, EAE, macrophages, multiple sclerosis, therapy

Date received: 14th December 2009; revised: 1st May 2010; 18th June 2010; accepted: 22nd June 2010

Introduction

Monocytes/macrophages infiltrating the central nervous system (CNS) in concert with activated resident microglia play a major role in the inflammatory neurodegenerative process of multiple sclerosis (MS). Considering the plethora of macrophage activation subsets and their complex roles in immune reactions,[1,2] there is growing evidence that macrophages play ambivalent roles in inflammation including MS development. Indeed, various types of monocyte and macrophage activation have been identified on the basis of their functions. *In vitro* studies show that they can be rapidly induced or reversed, depending on applied stimuli.[3–5]

Classically activated M1 pro-inflammatory macrophages are typical in inflammatory reactions and in pathogen-defense; they can be induced by e.g. LPS, IFNγ, or TNFα, which leads to the expression of pro-inflammatory cytokines and cell markers. The term 'alternative activation (M2)' gathers together a family

[1]University Victor Segalen, France.
[2]CNRS UMR 5536, RMSB, France.

Corresponding author:
Claudine Boiziau, EA2966 Neurobiology of Myelin Disorders Laboratory, University Victor Segalen Bordeaux 2, France.
Email: claudine.boiziau@inserm.fr

of three major 'non-inflammatory' macrophage phenotypes promoting T_H2-type responses associated with tumor progression, some parasite infections,[6–9] tissue repair, and debris removal.[1,2,10]

In both MS and experimental animal models, intracerebral M1 cells[11,12] as well as M2 subsets[13,14] were detected. The latter are usually described as foamy macrophages that acquire a distinctive morphology by the ingestion of myelin-derived lipids and efficiently produce anti-inflammatory cytokines.[13] Other activation markers were also detected in MS brain lesions, mostly with respect to chemokine receptors.[15,16]

The recently developed *in vivo* monitoring of macrophage CNS infiltration in MS by magnetic resonance imaging (MRI) with ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles contrast agents offers better possibilities to characterize cellular inflammatory reactions.[17–19] In relapsing experimental autoimmune encephalomyelitis (EAE) animal models of MS, MRI-USPIO examination at clinical onset allows prediction of relapsing clinical severity and loss of myelin and axons,[20] and serves as a useful tool in preclinical therapeutic evaluation.[21–23] Interestingly, in the CNS of EAE rats negative for MRI-USPIO, macrophage infiltration is still observed. Such data raise further questions on the heterogeneity of infiltrating macrophages having ambivalent functions in promoting demyelination and neurodegeneration versus neuroprotective repair mechanisms.

Even though monocytes circulating in the peripheral blood are easily accessible, very few studies focused on their phenotypes in MS patients.[24–26] Studies of therapeutic effects on immune cells showed that the immunomodulatory agent glatiramer acetate exerts its action on monocytes by diminishing their responsiveness to pro-inflammatory stimuli and bias it towards T_H2-type responses.[27,28]

The heterogeneity of activated pro-inflammatory versus immunomodulating monocytes/macrophages in MS pathology raises the question of their respective roles in inflammatory CNS lesions as factors propagating disease process or inducing and maintaining recovery. We hypothesized that not only the suppression of pro-inflammatory, but also the reconstitution of immunomodulatory, monocytes/macrophages is necessary to establish recovery. In the relapsing EAE rat model, we demonstrate that the balance modulation between pro-inflammatory and immunomodulatory monocyte/macrophage phenotypes at the early clinical phase is decisive for induction of a monophasic (mild) or relapsing (severe) EAE. In addition, therapeutic administration of M2-activated monocytes during ongoing first clinical attack suppresses relapsing EAE development. Our data show that M2-activated monocyte phenotype is suppressed during experimental neuroinflammation of relapsing EAE. The experimental restoration of M2 monocytes underlines the importance of M2-activation phenotype for recovery and shows that the discovery of molecules acting *in vivo* to increase the M2 phenotype creates an original research direction, by opening up new perspectives for therapeutic applications in MS.

Material and methods

Animals

Female Dark Agouti rats (Harlan, France), 7–8 weeks old, were immunized with spinal cord homogenate (100 mg) in incomplete Freund's adjuvant (100 µl) by subcutaneous injection into the tail base.[29] Animals were followed every day post-immunization for weight gain/loss and clinical score. Clinical symptoms were evaluated according to the scale: 0 – no clinical signs, 1 – flaccid tail, 2 – weakness of hind limbs/walking difficulties, 3 – paralysis of one hind limb, 4 – paraplegia, 5 – moribund/death. All experiments were performed in accordance with the European Union (86/609/EEC) and the French National Committee (87/848) recommendations (animal experimentation permission, France 33/00055).

MRI experiment. In a first series of experiments, at the onset of clinical symptoms (score 1) animals received an intravenous injection of USPIO nanoparticles (100 µl/100 g body weight), and a T2-weighted MRI examination was performed 24 hours later. Animals with signal abnormalities were classified as 'MRI+' and animals that did not were designated as 'MRI−'. To characterize further stages of disease with the MRI technique, 12 rats identified as positive ($n=8$) or negative ($n=4$) at the first MRI ('onset') received a second USPIO injection 2 days later in the middle of the first attack ('1st attack') and a third at the beginning of 'relapse' or an equivalent time point.

Histological brain analysis. In a second series of experiments, animals ($n=33$) were immunized and examined by MRI with USPIO injection at clinical onset. After MRI classification, they were randomly grouped and euthanized at four time points during EAE development: at disease onset ($n=8$), during the first attack ($n=8$), during relapse ($n=8$, severe EAE) and during recovery phase ($n=9$).

Blood analysis. In a third series of experiments, animals ($n=12$) were immunized and classified as described above. Blood samples (500 µl) from the tail vein were collected with K3-EDTA (15%)-rinsed syringes (Sigma, France) under gaseous anesthesia

(halothane) at five time points of disease: 'day 0' before immunization, 'onset', 3 days later (equivalent to '1st attack'), at 'relapse' or equivalent time ('recovery' for mild disease) and at 'recovery' after relapse in MRI+ animals.

Treatment with M2-activated monocytes. In the fourth series of experiments, rats were immunized and classified by MRI with USPIO injection at clinical onset as described above. Rats with a MRI+ signal and confirmed severe clinical disease with scores 3 or 4 ($n = 9$) were randomly grouped to receive at the third and fifth day of clinical disease two injections (2.5×10^6 cells/injection) of either M2-activated cells ($n = 5$) or of non-stimulated M0 cells ($n = 4$) serving as controls. Clinical development was monitored and animals were euthanized on the third day after the second cell injection.

Immunostaining of rat brainstem sections

After euthanizing and perfusion with 4% paraformaldehyde, rat brain tissue was recovered. Tissue blocks were sectioned with a vibratome (Leica, France). Serial free floating coronal sections, 30 µm thick (between four and nine sections covering 3 mm), were collected in Phosphate Buffer Saline (PBS). For immunohistochemistry, non-specific staining was blocked with 3% normal goat serum (Dako, France) in PBS containing 0.3% Triton X100 and sections were incubated over 72 hours at 4°C with primary antibodies: mouse monoclonal antibody against rat ED1 antigen (1/1000 dilution; Serotec, France) and rabbit polyclonal antibody against iNOS (1/2000 dilution; BD Biosciences, France) or rabbit polyclonal antibody against bovine arginase 1 (1/100 dilution; Abcam, France). Sections were then incubated with secondary antibodies: goat anti-mouse IgG Alexa® 488-conjugated antibody (1/1000 dilution; Molecular Probes, France) and goat anti-rabbit IgG CY3-conjugated antibody (1/1000 dilution; Jackson, USA). The analysis was performed using epifluorescent (Nikon, France) and confocal microscopy (Leica DMR TCS SP2 AOBS on an upright stand, using objectives HCX Plan Apo CS 40X NA 1.25). In the M1 and M2 analysis in severe and mild EAE, ED1+, M1 and M2 cells were counted by experienced researcher and normalized to mm^3 of CNS tissue. In the treatment study, microscopy images (100 x magnification, Nikon France) were automatically treated with Adobe Photoshop CS4 software to measure ED1+ labeled surfaces, and iNOS/Arginase 1 intensity colocalized with ED1+ surfaces. Therefore, this analysis gives a number of pixels (per section), whose fluorescence is above a pre-determined background threshold.

Isolation of circulating blood monocytes

Rat blood samples (500 µl) were collected from the tail vein. Peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll protocol (Eurobio, France) and purified by adhesion in RPMI 1640 medium. Purity of monocyte cultures was >95% as confirmed by ED1 immunostaining. Obtained cells were analysed for further gene expression characterization without any additional treatment.

Reverse transcription of mRNA isolated from adherent PBMC

After adhesion of monocytes recovered from the 500 µl blood samples (see above), total RNA extraction from adherent cells was performed using the RNeasy Micro Kit (Qiagen, France) according to the manufacturer protocols for monolayer cell cultures. After concentration by precipitation RNA was reverse-transcribed using Sensiscript® Reverse Transcriptase (Qiagen, France) and a dT18 oligonucleotide as primer.

Real-time PCR

The primer sequences are shown in Table 1. Efficiencies ≥85% were accepted in the study. Expression of genes was normalized to a reference gene – glyceraldehyde 3-phosphate dehydrogenase (GAPDH).[30] Real-time PCR was done using QuantiTect SYBR® Green PCR (Qiagen, France). The protocol was as follows: initial activation step (15 min at 95°C), cycle step (x 40): denaturation (15 s at 94°C), annealing (30 s at 55°C), extension (30 s at 72°C) and data acquisition (15 s at 72°C).

Activation of isolated blood monocytes

Whole blood was collected from healthy Dark Agouti female rats by heart puncture and circulating blood monocytes were isolated by standard Ficoll protocol. Adherent cells were recovered and cultured at the density of 10^6 cells/ml of culture medium for 20 h in low-attachment wells in RMPI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1% penicillin/streptomycin solution (complete medium) either in the presence of cytokines promoting M2 activation: interleukin-10 (20 ng/ml) and interleukin-13 (20 ng/ml), or in the absence of any cytokines ('M0' activation). To confirm the immunomodulatory M2-profile of collected cells, gene expression was analyzed as described above. Expression of iNOS and IL1β (pro-inflammatory M1 markers) on the one hand and Arg1 and TGFβ (M2 markers) on the other hand were measured by real-time quantitative RT–PCR in

Table 1. Primer sequences used in real-time PCR. Real-time PCR was done using QuantiTect SYBR® Green PCR on an i-Cycler (Bio-Rad) with the following protocol: initial activation step (15 min at 95°C), 40 cycles: denaturation (15 s at 94°C), annealing (30 s at 55°C), extension (30 s at 72°C) and data acquisition (15 s at 72°C). PCR product sizes (last column) were controlled by electrophoresis.

Gene	Primer sequences 5′–3′	Product size
GAPDH	sens: TGCTGGTGCTGAGTATGTCGTG	101 pb
	revs: CGGAGATGATGACCCTTTTGG	
iNOS	sens: GATTTTTCACGACACCCTTCACC	105 pb
	revs: GGTCCTCTGGTCAAACTCTTGGAG	
IL1β	sens: AATGACCTGTTCTTTGAGGCTGAC	115 pb
	revs: CGAGATGCTGCTGTGAGATTTGAAG	
Arginase I	sens: TGAACCCAACTCTTGGGAAG	178 pb
	revs: GTGATGCCCCAGATGACTTT	
IL10	RN_IL10_1SG_ bQuantitect Primer assay: sens + revs (Qiagen, France)	69 pb
CCL22	sens: GGCAGGAAGGACCATACAAA	200 pb
	revs: TCCAGAGGAGCAAGCAGATT	
TGFβ	sens: CCGCAACAACGCAATCTATG	180 pb
	revs: AGCCCTGTATTCCGTCTCCTT	

M2-activated cells and compared to M0 non-stimulated cells serving as a control. As expected, iNOS and IL1β expressions were identical or sixfold lower, respectively, whereas Arg1 and TGFβ were 14- and 3.5 fold more expressed in M2 cells, compared to M0 cells. After the labeling of cell membranes with PKH67 according to the manufacturer's protocol (Sigma, France) and extensive washing, 2.5×10^6 activated cells were injected intravenously in animals in 0.9% NaCl.

USPIO internalization in brain and MRI evaluation

The USPIO contrast agent was AMI-227 (Sinerem®, kindly given by Guerbet laboratory, Aulnay, France). Imaging studies were performed under general anesthesia (2% isoflurane). MRI of brain and upper spinal cord was performed at 4.7 T on a Brucker Biospec imager using a custom-made birdcage type head coil of 5 cm diameter. MRI examinations were performed with coronal spin echo T2 weighted RARE (Rapid Acquisition with Relaxation Enhancement) sequence (TR: 4200 ms and TE: 67.4 ms) and sagittal spin echo T2 weighted RARE sequence (TR: 3500 ms and TE: 67.4 ms). The following parameters were applied: 16 (coronal) or 12 (sagittal) contiguous slices, matrix size: 128×128, Field Of View = 3.25 cm × 3.25 cm, section thickness = 1.5 mm. In order to give an arbitrary quantitative estimation of MRI signal alterations with USPIO, the number of pixels presenting signal abnormality was quantified using Image J software, for each animal on all coronal sections per MRI scan covering the entire upper spinal cord and brain. The addition of the values gave the amount of signal alterations per animal at a given time point. Quantitative reading was performed independently by at least three experienced investigators and neuroradiologists.

Statistical analysis

Statistical analysis was done using Statview software following *Nature* recommendations (www.nature.com/nature/authors/gta). Median value (first quartile, third quartile) was used for data analysis of small groups. Differences between groups were evaluated using either Kruskall–Wallis (for at least three compared groups) or Mann–Whitney tests (for two compared groups) for non-parametric measures. To analyze multiple samples of blood the follow-up study Friedman test was used; the comparison of paired values was done with the use of the Wilcoxon matched-pair test or an analysis of variance (Anova) for repeated measures test and the Spearman test was used for correlation analysis. Two-tailed p values less than .05 were considered as significant.

Results

Monocyte infiltration in EAE is a spreading and non-continuous phenomenon

We investigated monocyte/macrophage expression patterns during inflammatory attacks in the relapsing EAE model in Dark Agouti rats. This model is characterized by inter-individual differences in disease development and severity[29] providing the opportunity of a comparative monocyte study between monophasic (mild) and relapsing (severe) EAE course, which can be predicted by MRI-USPIO at onset of clinical disease.[20]

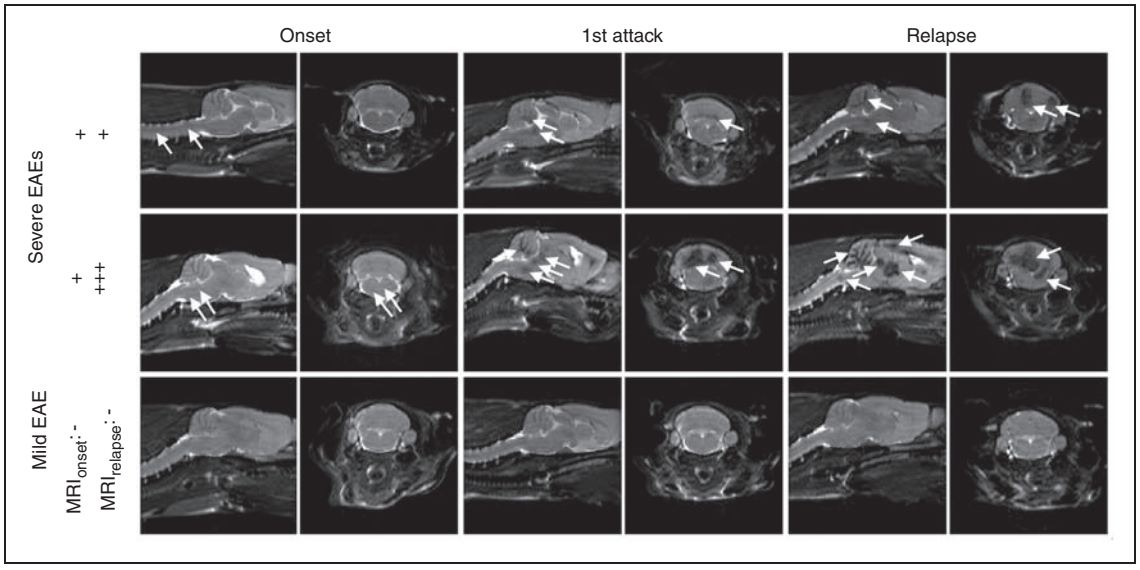


Figure 1. T2 W images of MRI follow-up of macrophage infiltration in EAE rat brains. 12 animals were injected with USPIO nanoparticles during several disease stages: at clinical onset (left panels), two days later at 1st attack (central panels), and at onset of relapse (right panels); two examples of 8 rats that developed severe EAE form (first and second lines) with hyposignals (white arrows) being detected at the clinical onset (MRI_{onset} +); one example of 4 animals showing no MRI signal changes during disease onset (MRI_{onset} −) and that had a mild EAE course (third line). On the second line a rat presenting very strong hyposignals during relapse ($MRI_{relapse}$ +++) is shown, as an example of extremely high USPIO load. All the MRI+ rats during MRI_{onset} were also + during $MRI_{1st\ attack}$ and most of them (7/8) during $MRI_{relapse}$. Medio-sagittal and coronal (brain stem-cerebellum level) views are shown for each rat and disease step.

Indeed, we previously demonstrated that rats showing MRI hyposignal at onset examination (MRI+) develop a relapsing EAE disease ('severe EAE'), in contrast with rats without any detected MRI hyposignal (MRI−) which have a monophasic EAE form ('mild EAE').[20]

First, to define the kinetics of monocyte/macrophage infiltration into the CNS of EAE rats, the MRI-USPIO technique was applied. The T2-weighted MRI method visualized intracerebral USPIO localization at three different disease timeframes: clinical onset, first attack and relapse or equivalent time for mildly sick animals. Monitoring of USPIO-loaded macrophages at different clinical stages showed that once animals show hyposignals at clinical onset, they remain MRI+ during the inflammatory stages of disease. Moreover, macrophage infiltration in the CNS of these animals spreads with disease progression (Figure 1): at onset, most of the lesions revealed by a T2 hyposignal were localized in the upper spinal cord and brainstem (median: 100% (72; 100) of the total lesion area) spreading into new brain areas, mostly cerebellum and sub-cortical regions at the peak of first attack (11% (0; 38) and 22% (0; 33) of total lesion load, respectively) and during relapse (Figure 1). In contrast, when USPIO was injected during the recovery phase, no or only minor signal abnormalities were observed (Petry et al., unpublished data). Therefore, the presence of persistent or newly occurring inflammatory lesions (upper spinal cord, cerebellum, and brainstem) throughout severe disease inflammatory phases was demonstrated. These data demonstrate that macrophage infiltration is time restricted and clearly depends on disease stage (e.g. onset versus recovery phase); moreover MRI abnormalities detected at each stage are associated with new infiltrations rather than remaining signals from previous USPIO injection. Despite the appearance of new inflammatory regions, the general lesion load as determined by total surface of MRI abnormalities was not increasing (p = NS; Friedman test).

Pro-inflammatory ED1+ cells accumulate around blood vessels, whereas ED1+ immunomodulating cells are dispersed in parenchyma

Having defined continuously inflamed CNS areas such as brainstem, we analyzed the activation profile of myeloid cells in these regions during four defined phases of disease. Based on our previous work demonstrating that MRI positivity at clinical onset is predictive for relapse occurrence,[20] in the second series of experiments all sick animals underwent MRI-USPIO examination and were classified as developing severe or mild EAE (MRI+ or MRI− respectively).

Activated myeloid cells in brainstem were identified by their expression of ED1 marker (ED1+ cells) characteristic for monocytes, macrophages, and activated microglia.[31,32] Activated ED1+ cells expressed either inducible nitric oxide synthase (iNOS, M1 marker) or

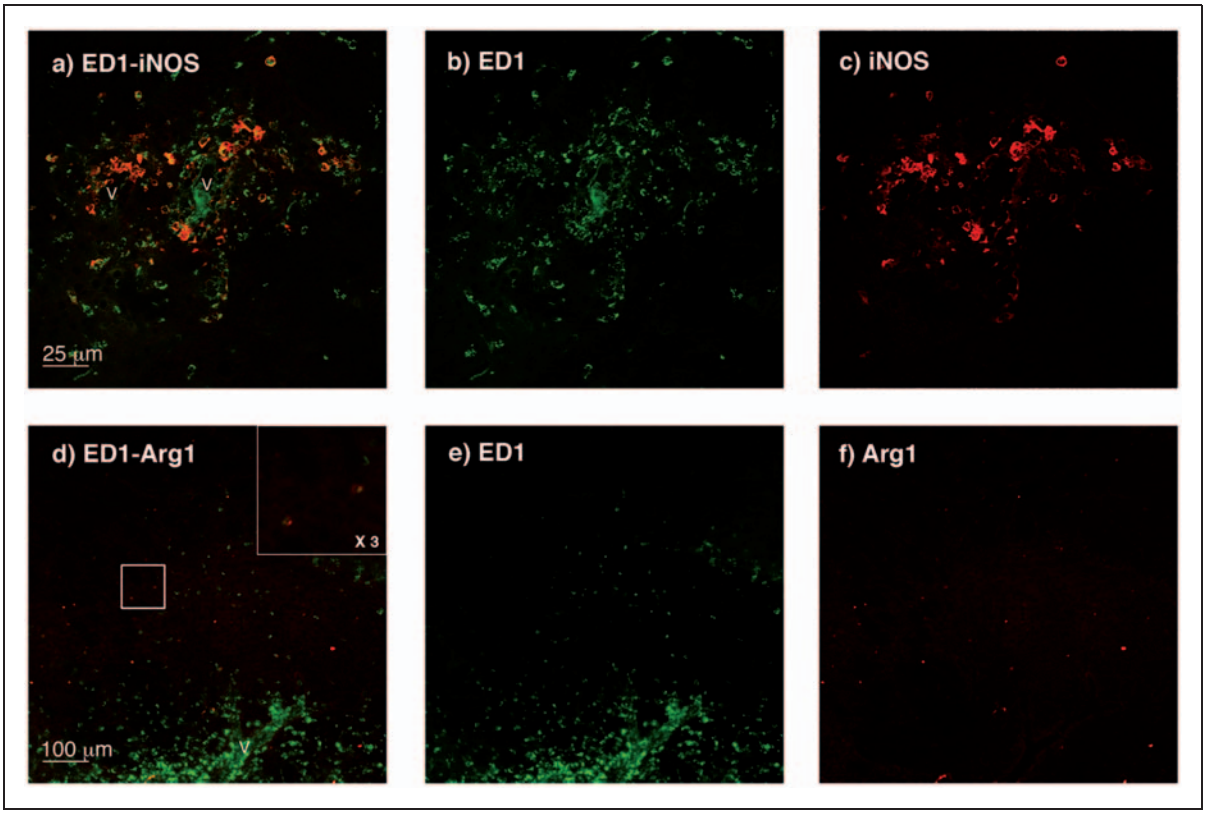


Figure 2. Activated M1 and M2 macrophages/microglia are present in brainstem tissue during relapsing EAE course in rat. Activated macrophages/microglia of rat brainstem sections were immunolabeled with mouse antibody against rat lysosomal membrane marker ED1 and visualized with a secondary antibody conjugated with Alexa®488 (green fluorochrome) (B and E). M1 (iNOS) (C) and M2 (Arg1) (F) cytoplasmic markers were revealed with rabbit polyclonal antibodies and visualized with a secondary antibody conjugated with Cy3® (red fluorochrome). M1 cells were mostly found around inflamed blood vessels ('V' in A), whereas M2 cells were scarce in these areas ('V', in D) and were more abundant at a certain distance from perivascular zones. Panels a and d show merged images with an enlargment (ED1-Arg1: X3) showing colabeled cells.

arginase 1 (Arg1, M2 marker) or neither of them (nonM1, nonM2). Immunolabeling of both marker enzymes that metabolize the same substrate, L-arginine allows the inclusion of a major proportion of the activated monocytes for analysis.

Immunostaining of brainstem tissue revealed that the majority of ED1+ cells were detected in perivascular spaces, accumulating around blood vessels (Figures 2A, D), but regions in the vicinity of the fourth ventricle also contained large zones of dispersed ED1+ cells (not shown). A subpopulation of these cells co-expressed iNOS protein (M1 phenotype) (Figures 2A–C). Apart from these cells, a smaller proportion of activated ED1+iNOS− cells dispersed in brain parenchyma was observed: when sections were co-labeled with anti-ED1 and anti-arginase 1 antibody, some dispersed ED1+ cells expressed mostly Arg1 (M2 phenotype) (Figures 2D–F). Therefore, in our model both M1 and M2 phenotypes are found throughout the brainstem, but are distributed differently in CNS tissue in respect to ongoing inflammatory reaction: M1 phenotype is mostly detected in perivascular space (>80% of ED1+iNOS+ are seen in these areas), and M2 phenotype as dispersed non-perivascular cells >90 % of ED1+Arg1+ cells).

Relapse is characterized by the suppression of immunomodulating M2 macrophages/microglia in lesion sites

In further steps, activated macrophages/microglia and the proportion of M1 (ED1+iNOS+ cells) and M2 (ED1+Arg1+ cells) myeloid cells were quantified in both severe (s, $n = 19$) and mild (m, $n = 14$) EAE animals at different stages of disease: at clinical onset, during first attack, relapse (for severe EAE animals)/ recovery (for mild EAE rats) and recovery after relapse in severe EAE. Animals were classified in each group based on MRI with USPIO nanoparticles performed at clinical onset for each individual rat. Analysis of CNS tissue revealed that the number of activated ED1+ cells remained almost constant during different acute EAE phases, however, as expected, severe EAE rats had significantly more ED1+ cells, compared to animals with

mild EAE (onset: $p = .021$; 1st attack: $p = .034$; relapse (s)/recovery (m): $p = .0012$) (Figure 3B). This effect, however, was no longer observed in recovering animals, when mild and severe EAE animals were compared in respect to clinical phase of disease (recovery of mild EAE versus severe EAE).

Phenotypes of activated macrophage/microglia were different in animals with different disease profiles. As the number of activated ED1+ cells in the CNS of mild and severe animals was very different, the characteristics of lesions (containing pro-inflammatory cells versus immunomudulatory ones) were evaluated by measure of the percentage of each inflammatory phenotype. In animals with mild monophasic EAE, ED1+ cell quantification revealed constant, very low numbers of activated cells located in the brainstem. Macrophage infiltration was very limited from the beginning of clinical symptoms until recovery. Interestingly, M1 and M2 macrophage proportions did not change between onset, first attack, and relapse and remained low (<10% of total ED1+ cells) (Figures 3C, D). Concerning the ED1+iNOS+ and ED1+Arg1+ cells ratio (M1/M2 ratio) during inflammatory phases, it remained constant and relatively low at onset, first attack, and recovery (between 0 and 3).

In contrast, animals with severe EAE (MRI+) showed variation in iNOS+ and Arg1+ activated macrophage/microglia proportions. In these animals about 40% of ED1+ cells expressed M1 phenotype at onset, and this value rapidly decreased during first attack (18%) and then remained stable during relapse phase (16%). During recovery, ED1+iNOS+ cells were hardly detectable in brainstems in comparison to the onset group (<1.5%) (Figure 3C). M2 macrophage proportion remained low. Yet in comparison to onset and first attack (about 3%), it was reduced even more during the second clinical episode (about 0.4%) ($p = .007$ and $p = .003$, respectively), indicating strong inhibition of M2 cell expression during the relapse (Figure 3D). Therefore the M1/M2 ratio did not change significantly between clinical onset and first attack (ratio about 12 and 6, respectively), although it was favoring a pro-inflammatory profile. During relapse, this imbalance was significantly increased (M1/M2 ratio = 40) in comparison to previous clinical stages ($p = .042$ and $p = .013$, respectively) (Figure 3E). These results show that relapse occurrence is associated with the inhibition of the intracerebral immunomodulating M2 cell profile.

Relapse occurrence is characterized by iNOS over-expression in circulating monocytes

As we observed suppression of immunomodulating M2 monocyte profile in the brainstem of EAE rats during relapse and an important presence of intracerebral M1 activated ED1+ cells in severely sick EAE rats, we further investigated expression profiles in circulating monocytes during inflammatory EAE phases.

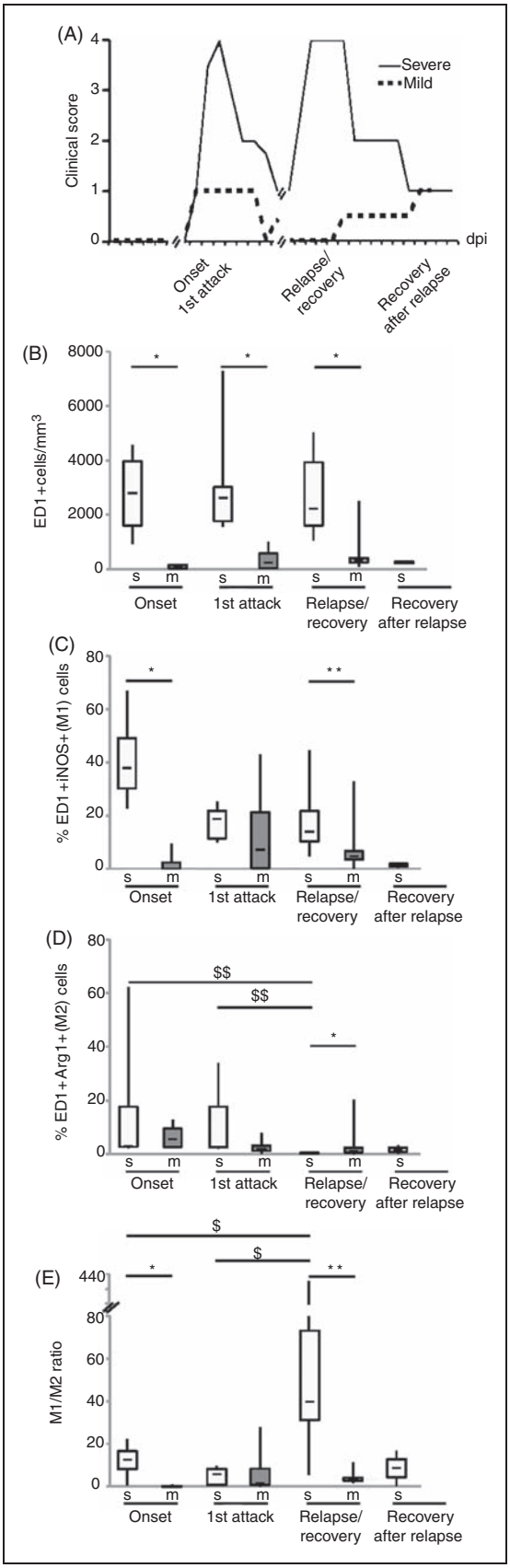
Animals ($n = 12$) were immunized as described before and classified into severe and mild EAE groups based on the maximal clinical score obtained individually (Figure 4A). Circulating monocytes were harvested from blood samples by Ficoll protocol and adhesion, and their global phenotypic activation status was evaluated by real time RT-PCR analysis of M1 (iNOS, IL1-β) and M2 (Arg1, IL10, TGFβ, CCL22) markers. Individual analysis of these genes showed stable expression levels (normalized to reference gene GAPDH) of M2-characteristic genes: Arg1 (Figure 4C) TGFβ, IL-10 or CCL22 (not shown), regardless of disease phase and severity. In contrast, some differences were detected regarding iNOS expression.

Contrary to the histological data of brainstem cell infiltration, equivalent iNOS expression in mild and severe EAE was noted during onset and at first attack (Figure 4B). However, whereas in mild EAE animals iNOS expression returned to low levels after first attack, this level remained significantly higher in severe relapsing EAE animals ($p = .0062$), resulting in a global monocyte profile biased towards pro-inflammatory M1 phenotype in comparison to mild EAE rats (ratio iNOS/Arg1: $p = .0446$ (Figure 4D), iNOS/TGFβ: $p = .0285$, iNOS/IL10: $p = .0062$, iNOS/CCL22: $p = .0275$ (data analysis not shown)). Interestingly, analysis of monocytes from the recovery phase of severe EAE animals showed that this ratio decreased to the values observed in the mild EAE group after first attack, but was very heterogeneous within the group, probably due to the various pace of inflammation resolution in individual rats (24.8 (21.1; 161.2) (Figure 4D). Similarly, iNOS expression showed a tendency to decrease after relapse in severe EAE group ($p = .068$) reaching the same level as observed in mild EAE animals after first clinical attack ($p = $ NS) (Figure 4B).

Treatment with M2-activated monocytes/macrophages suppresses EAE

To unequivocally confirm the functional and clinical relevance of the regulatory imbalance of monocyte M1/M2 phenotypes and consequent deficiency in M2 monocytes in the CNS and blood during acute phase and relapse as a disease-promoting factor, we modulated M2 cell expression and investigated the effect of administration of M2-activated monocytes in ongoing severe EAE disease.

For this treatment experiment only immunized rats presenting as MRI+ at onset of clinical disease were considered. The MRI+ defined animals were



randomly grouped for treatment with M2 or M0 (non-activated) monocytes. Until the beginning of treatment the two groups were identical concerning their clinical scores ($t_7 = -4.23$; $p = .68$). Treatment consisted of intravenous administration of 2.5×10^6 monocytes isolated from the blood of healthy syngenic Dark Agouti rats and cultured *ex vivo* either under activation conditions with IL-10/IL-13 (20 ng/ml each) to obtain M2-activated cells, or non-stimulated M0 cells (controls) on the third and fifth days of clinical disease. As in the previous experiment, during the three days separating onset and first attack analysis of severely sick rats, an increase of pro-inflammatory markers was detected in blood monocytes (Figure 4B), two monocyte injections were programed, each separated by two days.

Already first therapeutic administration of M2-stimulated cells resulted in a strong clinical effect in M2-treated animals compared to control animals (Figure 5A) with clinical scores showing significant differences from the first day post injection (group effect: $F(1,7) = 13.03$, $p = .0086$) and over time (time effect: $F(5,35) = 4.92$, $p = .0039$). While EAE rats after the second administration of M2-activated cells maintained stable, low clinical scores for several days, in control EAE rats short recovery was followed by a relapse as commonly observed in the relapsing EAE model in Dark Agouti rats (euthanized on the eighth day of clinical disease which allowed us to see only the beginning of relapse in M0-treated rats). There was no significant interaction between time and group ($F(5,35) = 1.01$, $p = .40$). Although 2.5×10^6 M2 monocytes were injected at each time point, no modification of the M1/M2 balance was observed in blood (not shown), indicating that intravenous injection of M2-activated monocytes does not change permanently the general circulating monocyte population.

Histological analysis of the CNS from treated animals euthanized 3 days after the second cell injection showed that fewer activated macrophages/microglia were detected in the brainstem of M2-treated rats compared to M0-treated animals, although the effect was not significant (Figure 5B). In addition, ED1+ cells in M2-treated rats had a higher expression level of arginase 1 ($p = .014$, Figure 5C), whereas iNOS expression was comparable in the two groups (not shown). These data demonstrated that the peripheral M2-treatment both diminished activation of macrophages/microglia in the brain and increased immunomodulatory properties of these cells. However, tracking of injected cells by their fluorescent membranes (labeling of cytoplasmic membranes was performed just before injection, see Materials and Methods section) did not allow localization of these cells in the inflammatory lesions (not shown).

Discussion

Despite extensive studies on MS patients and experimental research on animal models, the mechanisms underlying the physiopathology of MS remain only partially identified. The interdependencies between inflammatory CNS infiltrates favoring demyelination and neurodegeneration versus neuroprotective mechanisms, especially in relapsing-remitting MS (RR-MS), are still poorly understood.[33,34] Even though the majority of patients receive immunosuppressive or immunomodulating treatments disease progression is only delayed in time.

The growing evidence of macrophage functional heterogeneity in different pathologies raises questions of their role in setting-up and maintaining chronic inflammatory reaction during MS. It was demonstrated on human and animal studies that glatiramer acetate leads to inhibition of monocyte pro-inflammatory responsiveness by orientation towards T_H2-type-like responses.[27,28] The expression profile of monocytes could be a key factor in the process and successful treatment approaches of MS.

The imbalance of M1/M2 monocytes as a key factor of inflammation severity is confirmed in our study of relapsing Dark Agouti rat, indicating that animals with mild and spontaneously recovering disease after a first clinical attack, display a relatively balanced expression of M1/M2 phenotypes both in brain lesions and peripheral blood (similar to basal level observed prior to immunization).

Figure 3. Quantitative evaluation of activated M1 and M2 macrophages/microglia present in brainstem tissue during relapsing EAE course in rat. ED1+ and either iNOS+ or Arg1+ cell quantification was performed at four different time points of disease (onset, 1st attack, relapse, and recovery). A few other cells expressing either iNOS or Arg1, were also detected, however, as they were ED1−, their further histological analysis was not performed. (A) The medians of clinical scores of severely sick (plain line) and mildly sick (dotted line) rats are given; the *x* axis corresponds to days post immunization (dpi); as onset and relapse steps are differently delayed in each rat, timescales were aligned at these both steps. (B) ED1+ cell number/mm3 of tissue in MRI+ ('s' severe EAE, white boxes, onset $n = 4$, 1st attack $n = 5$, relapse $n = 8$, recovery after relapse $n = 2$) and MRI−('m' mild EAE, grey boxes, onset $n = 4$, 1st attack $n = 3$, recovery $n = 7$) animals. Percentage of M1 macrophages (ED1+iNOS+) (C) and M2 macrophages (ED1+Arg1+) (D) among all ED1+ cells. (E) proportion of M1 and M2 macrophages in brainstem tissue, presented as iNOS/Arg1 ratio. Statistics: * shows significant differences between severe and mild EAE rats at the same step of disease, and $ between rats of the same group (either severe or mild) and sacrificed at different time points; p values: * or $: $p \leq .05$, ** or $$: $p \leq .01$.

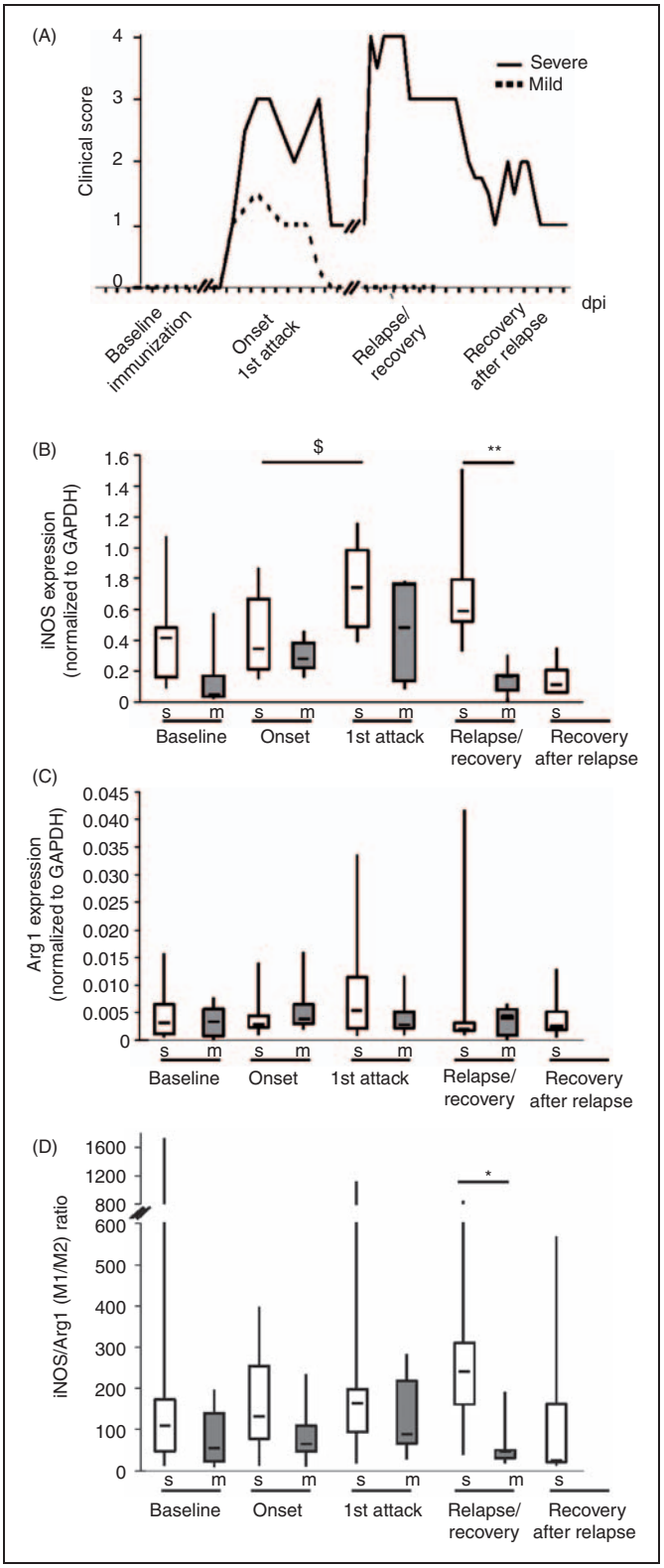


Figure 4. Comparison of iNOS, Arg1 expression and iNOS/Arg1 ratio in blood monocytes at different time points of severe and mild EAE. Adherent PBMC were purified from blood samples by Ficoll protocol and adhesion. Animals were classified into severe EAE ('s', white boxes, $n = 6$) or mild EAE group ('m', gray boxes, $n = 6$) based on their maximal clinical score: severe (≥ 3) and mild (≤ 2). iNOS and Arg1 expression in adherent PBMC were measured by real time RT-PCR and normalized to GAPDH expression. (A) Clinical scores (median values) of severely sick (plain line) and mildly sick (dotted line) rats aligned to the clinical onset and relapse onset; the *x*

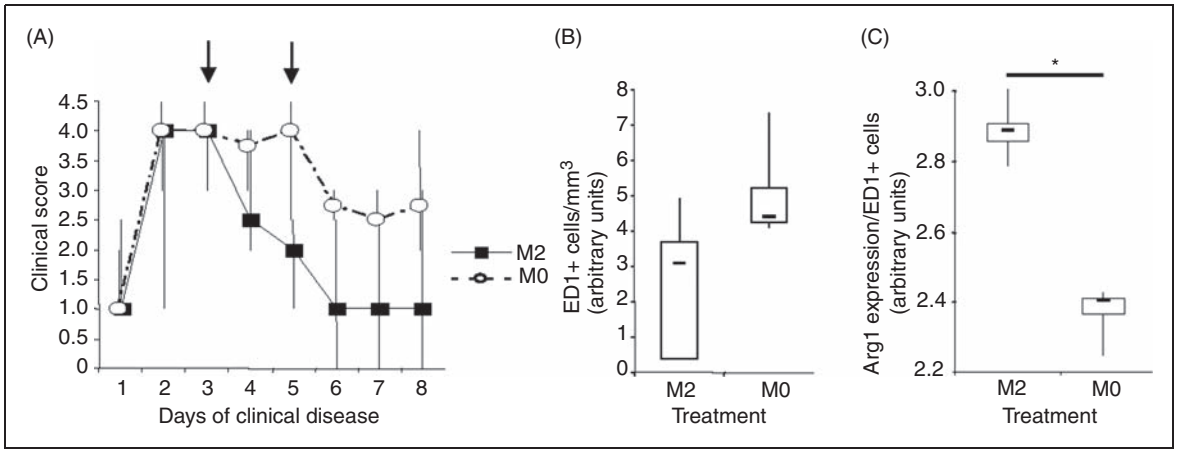


Figure 5. *Ex vivo* activated macrophages suppress EAE in severely sick rats. Animals with severe EAE course (based on MRI+ at clinical onset) reaching clinical scores 3 or 4 within the first 3 days after disease onset received two intravenous injections with M2-activated (M2) ($n = 5$) or M0 non-stimulated (M0) ($n = 4$) cells purified from blood and cultured as described in the Materials and Method section. M2-treated rats developed less severe disease, with lower clinical score after initiation of treatment in comparison to control M0-treated animals (A). Clinical scores expressed as median values with 1st and 3rd quartiles. Arrows: time points of injections. (B) Activated macrophages/microglia of rat brainstem sections were immunolabeled with anti-ED1 antibody. ED1+ cell area/mm^3 tissue as automatically measured with Adobe Photoshop CS4 software, is slightly decreased in M2-treated rats compared to M0-treated animals. (C) Expression level of arginase 1 was increased in ED1+ cells of M2-treated rats (median values (arbitrary units): M0-treatment: 2.40; M2 treatment: 2.89; $p = .014$): ED1+ surface (green layer) was used to determine a mask of activated macrophages/microglia; Arg1 signal was then retrieved as the histogram of the corresponding red layer (Adobe Photoshop CS4 software).

Relapse occurrence on the other hand, is characterized by strong pro-inflammatory bias. Furthermore, we demonstrated that the administration of *ex vivo* skewed M2a+c monocytes (activated with IL-10 and IL-13) efficiently suppresses clinical EAE, although no further analysis of stimulated M2 phenotype subfamilies[2,35,36] was performed in the present study.

M2 immunomodulating myeloid cells were already described in MS and EAE lesions,[11,13,14] and it also was shown that CNS inflammatory challenge stimulates microglial cells to activate mechanisms protecting CNS tissue.[37,38] In the light of these results, dispersed Arg1+ cells, that we observed in our model, could be considered as an activation step of permanently active surveying microglia,[39] suggesting that CNS cells themselves activate mechanisms to locally limit inflammation and prevent damage. After clinical recovery of EAE rats due to M2-activated cell injection, we expected a change in the brain cellular content, in comparison with M0-injected rats. Histological analysis demonstrated that general activation status of macrophages/microglia (ED1+ cells) showed a tendency to diminish in brainstem of M2-treated rats, although not significantly. Moreover, the immunomodulatory phenotype of activated macrophages/microglia was enhanced which in the presence of stable iNOS expression led to a change in proportion of M1 and M2 activated myeloid cells. In our opinion, this phenomenon could correspond to lower clinical scores observed in M2-treated animals. However, as no injected M2 cells were detected in brain lesions, we hypothesize that injected M2-activated monocytes acted in a peripheral compartment (like spleen or liver) but the precise target of their action remains to be identified. Indeed, as described by Wang et al.[40] in a mouse model of renal inflammation, intravenously injected M1- or M2-activated monocytes were detected in the spleen and liver 24 hours after treatment, and were still present in these tissues 14 and 28 days later. Moreover, *ex vivo* IL10/TGFβ-activated macrophages modified renal endogenous macrophage activation and successfully induced regulatory T-cell population *in vivo*, which was accompanied by reduced clinical symptoms in this model.[41] Whether similar processes take place in the peripheral compartment in our relapsing-remitting model of MS remains to elucidate.

Figure 4 (continued)
axis corresponds to days post immunization (dpi). Stable Arg1 expression was observed at all studied phases, both in severe and mild EAE (C). However, iNOS expression was differently regulated in severe and mild EAE groups (B). Its level remained stable in mild EAE, whereas in severe EAE rats it increased during 1st attack versus onset (\$, $p = .028$) and remained high during relapse versus low level in mild EAE rats (**, $p = .0062$), resulting in pro-inflammatory imbalance of monocyte profile (D) (*, $p = .045$). p values: \$ or *: $p \leq .05$; **: $p \leq .01$.

In the last two decades evidence has accumulated proving that the CNS and peripheral immune system are in permanent communication, and act together to restore homeostasis.[42–44] Indeed, intracerebral IL-1β injection promoted relatively instant leukocyte recruitment into the liver and systemic chemokine release as long as intracerebral inflammation was not resolved.[45,46] In mouse EAE, glatiramer acetate promotes *ex vivo* development of type-II activated monocytes, which reversed EAE when passively re-injected in recipient animals.[27,47]

In our model, some discrepancies in monocyte/macrophage activation phenotype occurred between the CNS and peripheral blood circulation. Similar pro-inflammatory imbalance of monocyte profile resulted from different phenomena. In the brainstem of severe EAE rats pro-inflammatory bias of macrophage profile came from a strong decrease of M2 (Arg1+) cell population, whereas in peripheral circulation it was associated with M1 increase (iNOS over-expression in monocytes). These data suggest that in this neuroinflammation model, immune reaction is differently regulated in various body compartments: the CNS and peripheral blood circulation. In a model of Lupus, studies on TGFβ expression and function in organ-specific autoimmunity demonstrated that while its level was decreased in peripheral circulation, its expression was increased in the target organ leading to the activation of regulatory T-cells and deregulated repair mechanisms.[48] It is possible that similar mechanisms are active in EAE autoimmunity, resulting in stimulation of peripheral inflammatory reaction and CNS microenvironment permissive to cell infiltration. Alternatively, recruitment into CNS lesions could be a selective process, favoring one cell phenotype, as this recruitment mechanism was observed in other pathologies.[49–51] This would explain the high dominance of M1-activated cells in perivascular regions of CNS tissue. Another hypothesis that should be verified is whether a shift of monocyte profiles takes place once they encounter the CNS microenvironment.

In experimental inflammatory chronic renal disease, M2 administration suppressed disease development.[40] Similarly in our study, we demonstrate the causality between disease gravity and M1/M2 disequilibrium and the curative effect by administration of M2-programed macrophages. However, in contrast with our results, Wang et al.[40] observed in their kidney inflammatory model that some injected monocytes (regardless of their phenotype M0/M1/M2) were detected in lesions, in addition to peripheral organs (liver, spleen, but not lung and heart). Interestingly, our attempts to provoke relapsing disease in MRI− animals by injection of M1-biased cells (activated *in vitro* by culturing in the presence of LPS (10 μg/ml) and INFγ (20 ng/ml)) were unsuccessful (not shown). These findings suggest that once the immune system is able to control immune challenge and maintain a balanced state of cell activation, it is resistant to further manipulations. Moreover, the negative impact of the disturbance in the equilibrium of M1/M2 macrophages might be a key element in the inflammatory process. The shift in phenotype proportions observed in rat EAE during relapse might be related to insufficient immuno-protective mechanisms that promote M2-phenotype expression.

Whether T-lymphocyte activation follows the same disequilibrium is a point that remains to be elucidated, however, some reports showed modified regulatory T-cells in relapsing-remitting MS patients.[52,53]

These observations are important in the light of the development of novel therapeutic agents, which could efficiently restore and stimulate factors involved in activation of M2 immunomodulating cell properties. Whereas intravenous injection of M2-activated monocytes might be difficult to organize for MS patients, we here demonstrate the concept that drugs favoring M2 differentiation are promising therapeutic approaches.

Acknowledgements

Claudine Boiziau and Klaus G. Petry equally directed this work. We thank Philippe Legros and Christelle Poujol (PICIN imaging centre at the *Bordeaux Neurosciences Institute*, France) for their expert technical help with confocal microscopy, Drs Tarik Touil, Olivier Anne, Vincent Dousset for their scientific help and discussions at the beginning of this work, Dr D. Nora Abrous for help in statistical analysis and Dr Derrick Robinson (Bordeaux, France) for correcting the manuscript.

Funding

Joanna Mikita was supported by a fellowship from French Minister of Research and Technology. Klaus G. Petry was supported by INSERM, University of Bordeaux 2, Conseil Régional d'Aquitaine, ANR-TecSan, French MS Research Society (ARSEP), and Prod-Innov.

Conflict of interest statement

None declared.

References

1. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A and Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 2004; 25: 677–686.
2. Mosser DM and Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008; 8: 958–969.
3. Porcheray F, Viaud S, Rimaniol AC, Léone C, Samah B, Dereuddre-Bosquet N, et al. Macrophage activation switching: an asset for the resolution of inflammation. *Clin Exp Immunol* 2005; 142: 481–489.

4. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK and Suttles J. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* 2005; 175: 342–349.
5. Gratchev A, Kzhyshkowska J, Köthe K, Muller-Molinet I, Kannookadan S, Utikal J, et al. Mphi1 and Mphi2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals. *Immunobiology* 2006; 211: 473–486.
6. Stein M, Keshav S, Harris N and Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 1992; 176: 287–292.
7. Mantovani A, Sozzani S, Locati M, Allavena P and Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002; 23: 549–555.
8. Kropf P, Fuentes JM, Fahnrich E, Arpa L, Herath S, Weber V, et al. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. *FASEB J* 2005; 19: 1000–1002.
9. Raes G, Beschin A, Ghassabeh GH and De Baetselier P. Alternatively activated macrophages in protozoan infections. *Curr Opin Immunol* 2007; 19: 454–459.
10. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003; 3: 23–35.
11. Oleszak EL, Zaczynska E, Bhattacharjee M, Butunoi C, Legido A and Katsetos CD. Inducible nitric oxide synthase and nitrotyrosine are found in monocytes/macrophages and/or astrocytes in acute, but not in chronic, multiple sclerosis. *Clin Diagn Lab Immunol* 1998; 5: 438–445.
12. Broholm H, Andersen B, Wanscher B, Frederiksen JL, Rubin I, Pakkenberg B, et al. Nitric oxide synthase expression and enzymatic activity in multiple sclerosis. *Acta Neurol Scand* 2004; 109: 261–269.
13. Boven L, Van Meurs M, Van Zwam M, Wierenga-Wolf A, Hintzen R, Boot R, et al. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. *Brain* 2006; 129: 517–526.
14. Chiang CS, Powell HC, Gold LH, Samimi A and Campbell IL. Macrophage/microglial-mediated primary demyelination and motor disease induced by the central nervous system production of interleukin-3 in transgenic mice. *J Clin Invest* 1996; 97: 1512–1524.
15. Trebst C, Sorensen TL, Kivisakk P, Cathcart MK, Hesselgesser J, Horuk R, et al. CCR1+/CCR5+ mononuclear phagocytes accumulate in the central nervous system of patients with multiple sclerosis. *Am J Pathol* 2001; 159: 1701–1710.
16. Trebst C, König F, Ransohoff R, Brück W and Stangel M. CCR5 expression on macrophages/microglia is associated with early remyelination in multiple sclerosis lesions. *Mult Scler* 2008; 14: 728–733.
17. Dousset V, Brochet B, Deloire M, Lagoarde L, Barroso B, Caille J, et al. MR imaging of relapsing multiple sclerosis patients using ultra-small-particle iron oxide and compared with gadolinium. *Am J Neuroradiol* 2006; 27: 1000–1005.
18. Vellinga MM, Oude Engberink RD, Seewann A, Pouwels PJ, Wattjes MP, van der Pol SM, et al. Pluriformity of inflammation in multiple sclerosis shown by ultra-small iron oxide particle enhancement. *Brain* 2008; 131: 800–807.
19. Stoll G, Bendszus M, Perez J and Pham M. Magnetic resonance imaging of the peripheral nervous system. *J Neurol* 2009; 256: 1043–1051.
20. Brochet B, Deloire M, Touil T, Anne O, Caille J, Dousset V, et al. Early macrophage MRI of inflammatory lesions predicts lesion severity and disease development in relapsing EAE. *Neuroimage* 2006; 32: 266–274.
21. Deloire MS, Touil T, Brochet B, Dousset V, Caille JM and Petry KG. Macrophage brain infiltration in experimental autoimmune encephalomyelitis is not completely compromised by suppressed T-cell invasion: in vivo magnetic resonance imaging illustration in effective anti-VLA-4 antibody treatment. *Mult Scler* 2004; 10: 540–548.
22. Floris S, Blezer EL, Schreibelt G, Döpp E, van der Pol SM, Schadee-Eestermans IL, et al. Blood-brain barrier permeability and monocyte infiltration in experimental allergic encephalomyelitis: a quantitative MRI study. *Brain* 2004; 127: 616–627.
23. Rausch M, Hiestand P, Foster CA, Baumann DR, Cannet C and Rudin M. Predictability of FTY720 efficacy in experimental autoimmune encephalomyelitis by in vivo macrophage tracking: clinical implications for ultrasmall superparamagnetic iron oxide-enhanced magnetic resonance imaging. *J Magn Reson Imaging* 2004; 20: 16–24.
24. Kouwenhoven M, Teleshova N, Ozenci V, Press R and Link H. Monocytes in multiple sclerosis: phenotype and cytokine profile. *J Neuroimmunol* 2001; 112: 197–205.
25. Kim HJ, Ifergan I, Antel JP, Seguin R, Duddy M, Lapierre Y, et al. Type 2 monocyte and microglia differentiation mediated by glatiramer acetate therapy in patients with multiple sclerosis. *J Immunol* 2004; 172: 7144–7153.
26. Furlan R, Rovaris M, Boneschi FM, Khademi M, Bergami A, Gironi M, et al. Immunological patterns identifying disease course and evolution in multiple sclerosis patients. *J Neuroimmunol* 2005; 165: 192–200.
27. Weber MS, Prod'homme T, Youssef S, Dunn SE, Rundle CD, Lee L, et al. Type II monocytes modulate T cell-mediated central nervous system autoimmune disease. *Nat Med* 2007; 13: 935–943.
28. Weber MS, Starck M, Wagenpfeil S, Meinl E, Hohlfeld R and Farina C. Multiple sclerosis: glatiramer acetate inhibits monocyte reactivity in vitro and in vivo. *Brain* 2004; 127: 1370–1378.
29. Lorentzen JC, Issazadeh S, Storch M, Mustafa MI, Lassman H, Linington C, et al. Protracted, relapsing and demyelinating experimental autoimmune encephalomyelitis in DA rats immunized with syngeneic spinal cord and incomplete Freund's adjuvant. *J Neuroimmunol* 1995; 63: 193–205.
30. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3: 34.

31. Dijkstra CD, Van Vliet E, Döpp EA, van der Lelij AA and Kraal G. Marginal zone macrophages identified by a monoclonal antibody: characterization of immuno- and enzyme-histochemical properties and functional capacities. *Immunology* 1985; 55: 23–30.
32. Damoiseaux JG, Döpp EA, Calame W, Chao D, MacPherson GG and Dijkstra CD. Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. *Immunology* 1994; 83: 140–147.
33. Lassmann H, Brück W and Lucchinetti CF. The immunopathology of multiple sclerosis: an overview. *Brain Pathol* 2007; 17: 210–218.
34. Muzio L, Martino G and Furlan R. Multifaceted aspects of inflammation in multiple sclerosis: the role of microglia. *J Neuroimmunol* 2007; 191: 39–44.
35. Mantovani A, Sica A and Locati M. New vistas on macrophage differentiation and activation. *Eur J Immunol* 2007; 37: 14–16.
36. Varin A and Gordon S. Alternative activation of macrophages: immune function and cellular biology. *Immunobiology* 2009; 214: 630–641.
37. Columba-Cabezas S, Serafini B, Ambrosini E, Sanchez M, Penna G, Adorini L, et al. Induction of macrophage-derived chemokine/CCL22 expression in experimental autoimmune encephalomyelitis and cultured microglia: implications for disease regulation. *J Neuroimmunol* 2002; 130: 10–21.
38. Ponomarev ED, Maresz K, Tan Y and Dittel BN. CNS-derived interleukin-4 is essential for the regulation of autoimmune inflammation and induces a state of alternative activation in microglial cells. *J Neurosci* 2007; 27: 10714–10721.
39. Hanisch UK and Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 2007; 10: 1387–1394.
40. Wang Y, Wang YP, Zheng G, Lee VW, Ouyang L, Chang DH, et al. Ex vivo programmed macrophages ameliorate experimental chronic inflammatory renal disease. *Kidney Int* 2007; 72: 290–299.
41. Cao Q, Wang Y, Zheng D, Sun Y, Wang Y, Lee VW, et al. IL-10/TGF-beta-modified macrophages induce regulatory T cells and protect against adriamycin nephrosis. *J Am Soc Nephrol* 2010; 21: 933–942.
42. Teeling JL and Perry VH. Systemic infection and inflammation in acute CNS injury and chronic neurodegeneration: Underlying mechanisms. *Neuroscience* 2008; [Epub ahead of print].
43. Rosas-Ballina M and Tracey KJ. The neurology of the immune system: neural reflexes regulate immunity. *Neuron* 2009; 64: 28–32.
44. Rosas-Ballina M and Tracey KJ. Cholinergic control of inflammation. *J Intern Med* 2009; 265: 663–679.
45. Campbell SJ, Perry VH, Pitossi FJ, Butchart AG, Chertoff M, Waters S, et al. Central nervous system injury triggers hepatic CC and CXC chemokine expression that is associated with leukocyte mobilization and recruitment to both the central nervous system and the liver. *Am J Pathol* 2005; 166: 1487–1497.
46. Campbell SJ, Deacon RM, Jiang Y, Ferrari C, Pitossi FJ and Anthony DC. Overexpression of IL-1beta by adenoviral-mediated gene transfer in the rat brain causes a prolonged hepatic chemokine response, axonal injury and the suppression of spontaneous behaviour. *Neurobiol Dis* 2007; 27: 151–163.
47. Tierney JB, Kharkrang M and La Flamme AC. Type II-activated macrophages suppress the development of experimental autoimmune encephalomyelitis. *Immunol Cell Biol* 2009; 87: 235–240.
48. Saxena V, Lienesch DW, Zhou M, Bommireddy R, Azhar M, Doetschman T, et al. Dual roles of immunoregulatory cytokine TGF-beta in the pathogenesis of autoimmunity-mediated organ damage. *J Immunol* 2008; 180: 1903–1912.
49. Lumeng CN, Bodzin JL and Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 2007; 117: 175–184.
50. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med* 2007; 204: 3037–3047.
51. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, et al. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J Clin Invest* 2007; 117: 185–194.
52. Venken K, Hellings N, Hensen K, Rummens J, Medaer R, D'hooghe M, et al. Secondary progressive in contrast to relapsing-remitting multiple sclerosis patients show a normal CD4+CD25+ regulatory T-cell function and FOXP3 expression. *J Neurosci Res* 2006; 83: 1432–1446.
53. Xu L, Xu Z and Xu M. Glucocorticoid treatment restores the impaired suppressive function of regulatory T cells in patients with relapsing-remitting multiple sclerosis. *Clin Exp Immunol* 2009; 158: 26–30.