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Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis

Leonie A. Boven,[1] Marjan Van Meurs,[1] Marloes Van Zwam,[1] Annet Wierenga-Wolf,[1] Rogier Q. Hintzen,[2] Rolf G. Boot,[4] Johannes M. Aerts,[4] Sandra Amor,[5] Edward E. Nieuwenhuis[3,*] and Jon D. Laman[1,*]

[1]Departments of Immunology and [2]Neurology, part of the multiple sclerosis Centre ErasMS, [3]Department of Pediatrics, Erasmus Medical Center, Rotterdam, [4]Department of Medical Biochemistry, Academic Medical Center Amsterdam, [5]Biomedical Primate Research Center, part of the multiple sclerosis Centre ErasMS, Rijswijk, The Netherlands

Correspondence to: Leonie A. Boven, PhD, Department of Immunology, Ee802, Erasmus MC, PO Box 1738, 3000 DR Rotterdam, The Netherlands
E-mail: l.boven@erasmusmc.nl
*These authors contributed equally to this work.

Multiple sclerosis lesion activity concurs with the extent of inflammation, demyelination and axonal suffering. Pro-inflammatory myeloid cells contribute to lesion development, but the self-limiting nature of lesions implies as yet unidentified anti-inflammatory mechanisms. We addressed the hypothesis that myelin ingestion by myeloid cells induces a foamy appearance and confers anti-inflammatory function. First, we show that myelin-containing foam cells in multiple sclerosis lesions consistently express a series of anti-inflammatory molecules while lacking pro-inflammatory cytokines. Second, unique location-dependent cytokine and membrane receptor expression profiles imply functional specialization allowing for differential responses to microenvironmental cues. A novel human *in vitro* model of foamy macrophages functionally confirmed that myelin ingestion induces an anti-inflammatory programme. Foamy macrophages are unable to respond to prototypical inflammatory stimuli but do express molecules involved in suppression of inflammation. These findings provide novel insights into the mechanisms of lesion control and may open new roads to intervention.

Keywords: autoimmunity; brain; chemokines; cytokines; inflammation

Abbreviations: MOG = myelin oligodendrocyte glycoprotein; NAWM = normal appearing white matter; ORO = oil red O; PGES = prostaglandin E_2 synthase

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Introduction

Multiple sclerosis is a chronic inflammatory autoimmune disease of the CNS and is characterized by the presence of demyelinated areas throughout the CNS (Sospedra and Martin, 2005). Various mechanisms leading to demyelination and axonal suffering have been implicated and the production of toxic inflammatory mediators by infiltrating and resident CNS macrophages is believed to play a pivotal role (Raine, 1994; Becher *et al.*, 2000; Wingerchuk *et al.*, 2001; Cannella and Raine, 2004; Lassmann, 2004; Matute and Perez-Cerda, 2005; Sospedra and Martin, 2005).

Different subsets of myeloid cells have distinct roles in the development of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. These distinct and specialized roles of myeloid cells depend on their origin and, importantly, their location (Greter *et al.*, 2005; Heppner *et al.*, 2005; McMahon *et al.*, 2005; Platten and Steinman, 2005). As such, perivascular cells appear to be optimally positioned for the modulation of infiltrating T cell activity whereas parenchymal myeloid cells may have a more prominent role in mechanisms involved in myelin breakdown and axonal suffering (Platten and Steinman, 2005).

The plasticity and functional polarization of macrophages have received renewed attention in the light of novel key properties of different forms of macrophages. Two extremes of a continuum have been identified for macrophages, being M1, or classically activated macrophages and M2, or alternatively activated macrophages (Mantovani *et al.*, 2002; Gordon, 2003; Mosser, 2003; Mantovani *et al.*, 2004). The M1 phenotype is typically induced *in vitro* by IFN-γ,

TNF-α or LPS, whereas the M2 phenotype can be induced by IL-10, IL-4 or by the lipid mediator PGE_2, which is a strong inhibitor of pro-inflammatory immune responses (Kalinski *et al.*, 1997; Hinz *et al.*, 2000; Gratchev *et al.*, 2001; Ikegami *et al.*, 2001; Harris *et al.*, 2002). M1 macrophages are characterized by a high production of pro-inflammatory mediators and are involved in Th1 cell responses and the killing of micro-organisms and tumour cells. In contrast, M2 macrophages are associated with Th2 responses, scavenging of debris, promotion of tissue remodelling and repair and expression of anti-inflammatory molecules, including IL-1ra (IL-1 receptor antagonist) and CCL18 (Gordon, 2003; Mantovani *et al.*, 2004). CCL18 in particular is a specific marker for human alternatively activated macrophages (Kodelja *et al.*, 1998; Goerdt *et al.*, 1999; Mantovani *et al.*, 2002; Gordon, 2003) and is involved in immune suppression (Gordon, 2003; Vulcano *et al.*, 2003). In contrast to *in vitro* studies, the M2 phenotype *in vivo* is more variable. An M2-like phenotype can be observed in various other situations, including helminth infection, cancer, Gaucher disease and in healthy lung tissue (Mantovani *et al.*, 2002; Boven *et al.*, 2004; Herbert *et al.*, 2004; Beharka *et al.*, 2005). However, variations in expression profiles of M2-markers are observed depending on specific cues. It seems likely that the different phenotypes result from different cues leading to the M2-like phenotype (e.g. different cytokine milieu, parasite phagocytosis, lipid accumulation, surfactant ingestion) enabling the cell to respond to and deal with the specific stimulus in an appropriate way.

Demyelinating multiple sclerosis lesions are characterized by the presence of foamy macrophages, a characteristic subset of myeloid cells, which acquire their distinctive morphology by ingestion and accumulation of vast amounts of myelin-derived lipids. Foamy macrophages originate from both resident microglia and infiltrating monocytes (Li *et al.*, 1996). Besides their apparent role in scavenging myelin, it is still poorly understood if and how foamy macrophages may affect the local inflammatory process. Since multiple sclerosis lesions are self-limiting and do not expand indefinitely it is likely that local mechanisms restrict CNS inflammation and may also promote tissue repair. We hypothesized that foamy macrophages are anti-inflammatory M2-type macrophages and actively contribute to the resolution of brain inflammation. Our findings reveal an important and

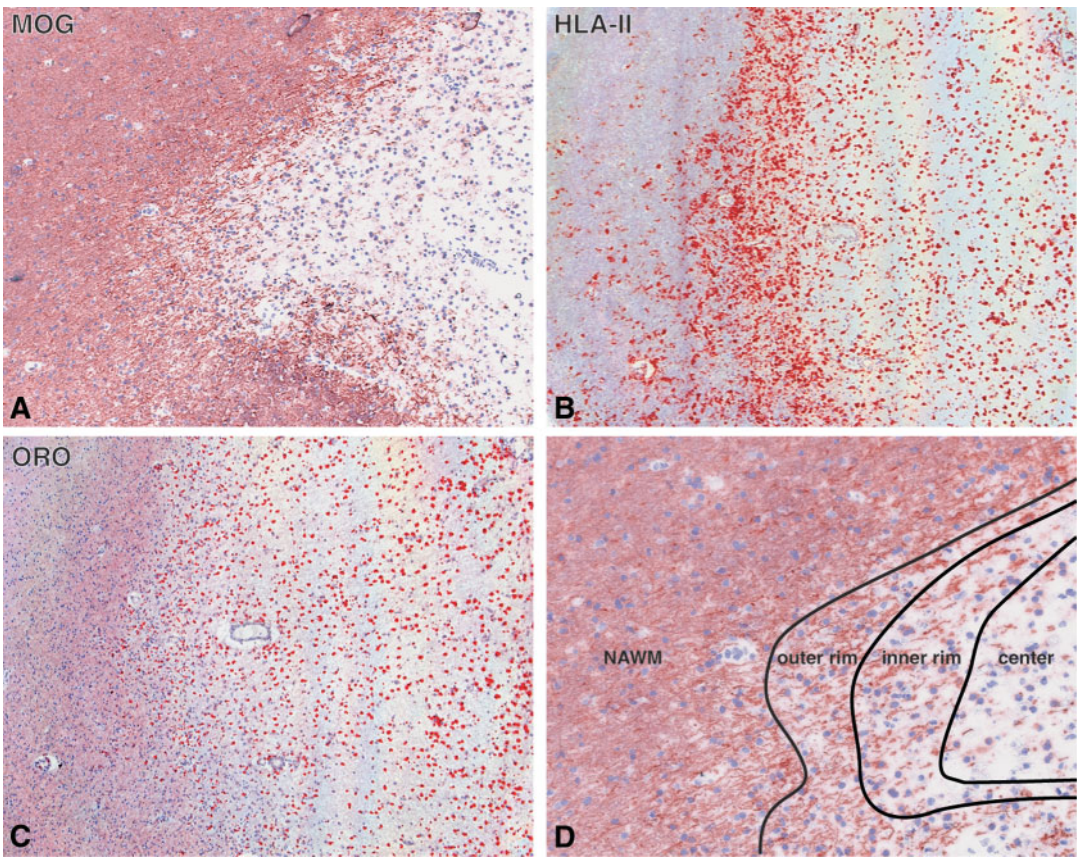


Fig. 1 Definition of micro-locations of foamy macrophages within active demyelinating multiple sclerosis lesions. (**A**) Extensive demyelination in post-mortem brain tissue of a multiple sclerosis patient as shown by the absence of MOG immunoreactivity in the lesion. Magnification ×8 (**B**) Strong HLA-II immunoreactivity is present on foamy macrophages throughout the lesion. Magnification ×8. (**C**) Foamy macrophages contain abundant neutral lipids as shown by ORO staining. Magnification ×8. (**D**) Based on the degree of demyelination as well as presence and size of ORO+ and HLA-II+ foamy macrophages arbitrary lines were set dividing NAWM, outer rim, inner rim and centre of the lesion. Magnification ×16.

previously overlooked anti-inflammatory role for foamy macrophages in multiple sclerosis lesions.

Material and methods

Immunohistochemical analysis of post-mortem multiple sclerosis brain tissue

Human autopsy brain tissue from 5 multiple sclerosis patients was provided by the Netherlands Brain Bank in Amsterdam (Coordinator Dr R. Ravid). Immunohistochemistry was performed on frozen sections of multiple sclerosis brain tissue to detect expression of (anti-)inflammatory markers and CNS antigens as described previously (Hoefakker *et al.*, 1995). In brief, 6 μm frozen sections were cut and thawed on to glass slides. Slides were kept overnight at room temperature in humidified atmosphere. After air-drying, slides were fixed in acetone containing 0.02% (v/v) H_2O_2. Slides were then air-dried for 10 min, washed with PBS and incubated with optimally diluted primary antibody overnight at 4°C in humidified atmosphere. Incubations with secondary rabbit anti-mouse-Ig-biotin (Dako) and tertiary horseradish peroxidase (HRP)-labelled avidin–biotin complex (ABC/HRP: Dako) were performed for 1 h at RT. HRP activity was revealed by incubation for 10 min at RT with 3-amino-9-ethyl-carbazole (AEC: Sigma), leading to a bright red precipitate. After washing, sections were counterstained with

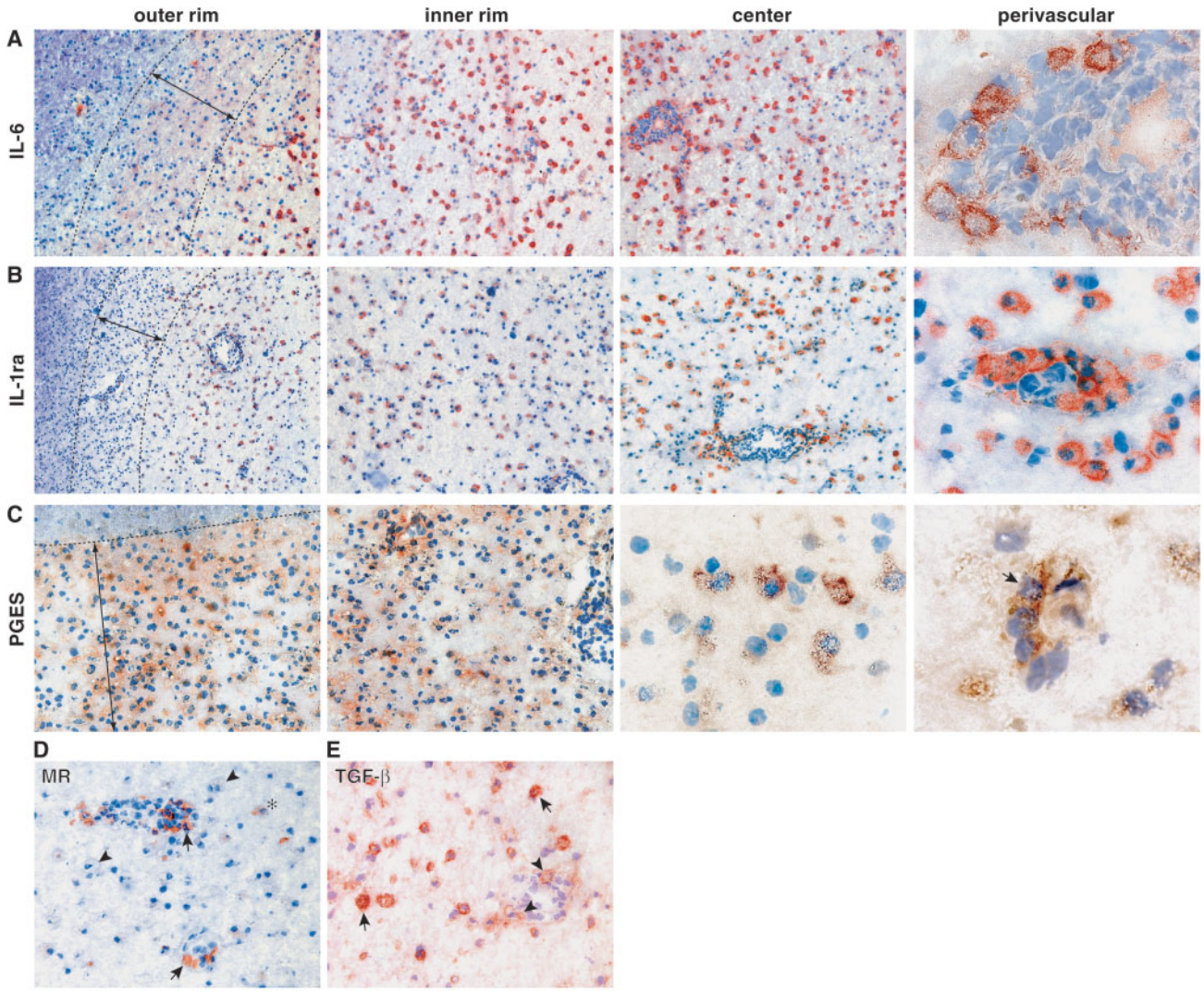


Fig. 2 Foamy macrophages in multiple sclerosis lesions display a unique microlocation-related phenotype and are anti-inflammatory. (**A**) IL-6 was not expressed by foamy macrophages in the outer rim (indicated by arrow between dotted lines), but was heavily expressed by foamy macrophages in the inner rim, lesion centre and in perivascular spaces. Magnification ×16, ×16, ×16, ×64. (**B**) Anti-inflammatory IL-1ra was expressed to a lesser extent by foamy macrophages in the outer and inner rim, but more pronounced in the lesion centre and most strongly by foamy macrophages in perivascular spaces. Magnification ×16, ×16, ×16, ×64. (**C**) PGES was present most intensely in foamy macrophages in the outer rim of the lesion and less pronounced in foamy macrophages in the inner rim, lesion centre or in perivascular spaces. Magnification ×32, ×32, ×32, ×64. (**D**) Foamy macrophages that are in close proximity, but in different micro-locations, differentially expressed molecules. Immunoreactivity for mannose receptor was mostly restricted to foamy macrophages in perivascular spaces (arrow) with an occasional positive parenchymal foamy macrophage (asterisk). Arrowheads indicate mannose receptor-negative foamy macrophages. The reverse was observed for TGF-β, which was more strongly expressed by foamy macrophages in the lesion (arrow) as compared with those in perivascular spaces (arrowhead). Magnification ×32, ×32.

haematoxylin and embedded with glycerol-gelatin. Omission of primary antibody acted as control staining. Myelin degradation products were detected with oil-red O (ORO), which stains neutral lipids, as previously described (Chayen and Bitensky, 1991). The antibodies used were directed against IL-1ra (Biosource), IL-4 (U-Cytech), PGES (Cayman), TGF-beta (Santa Cruz) and CCL18 (R&D) as anti-inflammatory markers; HLA class II (Dako), CD163 (gift from Prof. C.D. Dijkstra), mannose receptor (gift from Dr F. Noorman), CD11b (BD Biosciences) for antigen recognition and presentation; IL-1β (gift from Dr Boraschi), TNF-α (U-Cytech), IL-6 (Genzyme), IL-12p40/p70 (Pharmingen); for CNS proteins MOG, MAP-2 (Pierce) as pro-inflammatory markers.

In vitro model for myelin-driven foam cell formation

Myelin was isolated as described previously (Norton and Poduslo, 1973). In short, white matter derived from post-mortem brain tissue (gift from Dr Hans van Noort) was homogenized in 0.32 M sucrose and subsequently layered on 0.85 M sucrose. After centrifugation at 75 000 *g* myelin was collected from the interface, washed in water and suspended in water for osmotic shock. Using this method, the purified myelin was shown to be free of any recognizable fragments of other subcellular elements. Previous studies have shown that purified myelin structurally resembled the whole myelin as seen in tissue sections using electron microscopy (Autilio *et al.*, 1964).

Peripheral blood mononuclear cells were isolated from heparinized blood from healthy donors using a Ficoll density gradient. Subsequently, monocytes were purified using Percoll density gradient resulting in >80% monocytes. Monocytes were cultured in suspension at a concentration of 1×10^6 cells/ml in Teflon flasks (Nalgene) in RPMI with 5% human AB serum. After 5–7 days monocyte-derived macrophages were recovered from the Teflon flasks and seeded in tissue culture plates. After 24 h non-adherent cells were removed and remaining cells were >95% macrophages as determined by macrophage-specific esterase staining. Foamy

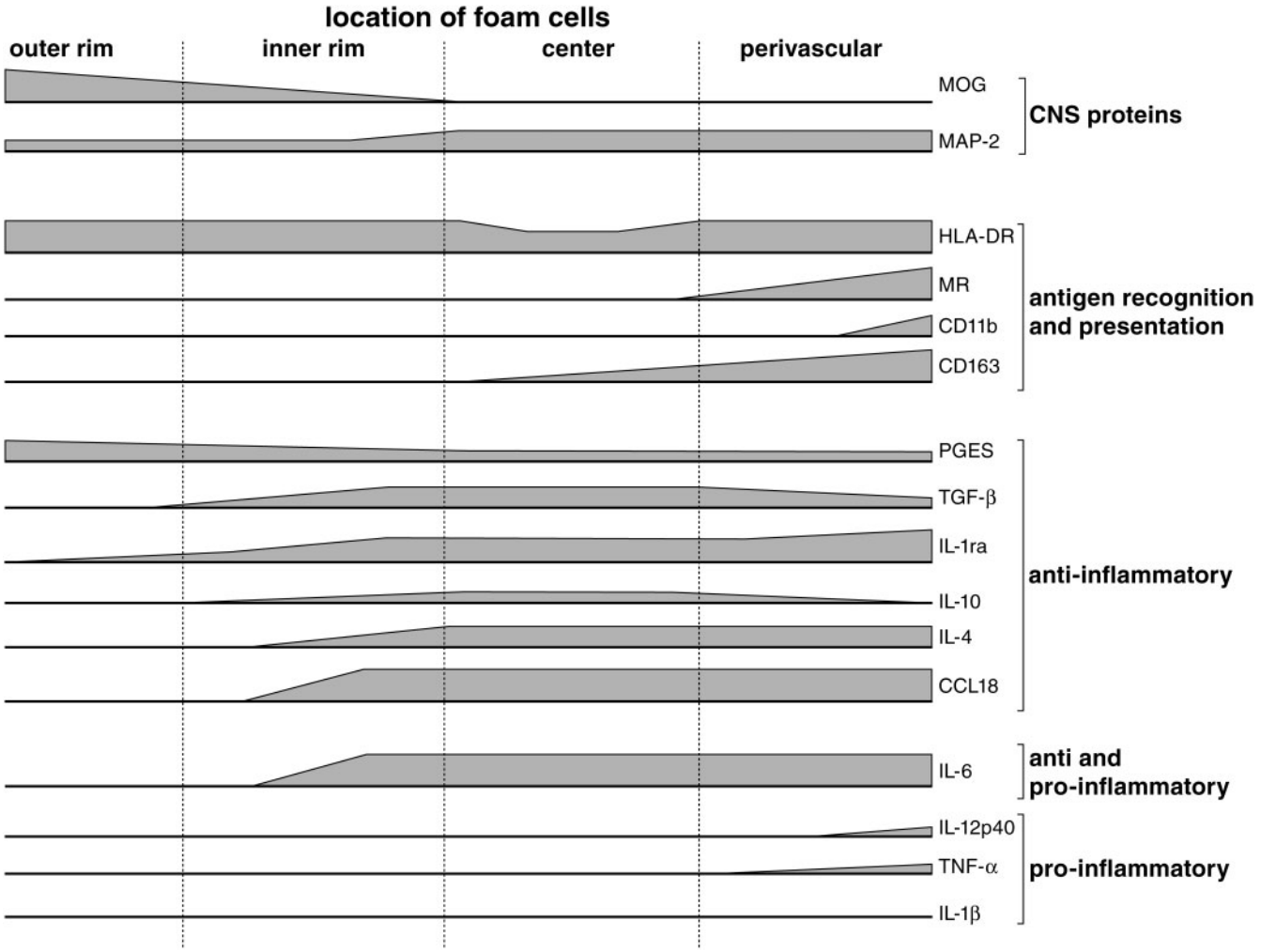


Fig. 3 Microlocation-dependent expression profiles of surface and intercellular molecules by foamy macrophages in multiple sclerosis lesions. The presence of CNS proteins, molecules involved in antigen recognition and presentation, as well as anti- and pro-inflammatory molecules was analysed for foamy macrophages at different sites within the lesion, i.e. in the outer rim, the inner rim, lesion centre and in perivascular spaces. These sites are indicated by dotted lines. Quantification was based on frequency of positive foamy macrophages and staining intensity and was performed on 2–3 lesions from 4 different multiple sclerosis patients. The gradient between lesion centre and the perivascular space reflects increasing or decreasing staining frequency and/or intensity towards the perivascular compartment.

macrophages were generated *in vitro* by incubating macrophages with myelin for 24 h-7 days (referred to as 1 day and 7 day-old foamy macrophages). For experiments shown in Fig. 5A the indicated concentrations of myelin were used, for all subsequent experiments 50 μg/ml myelin was used. Control macrophages were obtained from the same donor and not fed with myelin.

ELISA

To determine cytokine production in culture supernatants of foamy macrophages commercial capture ELISA was performed. TNF-α, IL-10 and IL-12p40 were measured in the collected culture supernatants. ELISA was performed according to the manufacturers' guidelines (Biosource). Briefly, polystyrene microtitre wells (Immuno Maxisorp) were coated overnight at 4°C with monoclonal anti-cytokine capture antibodies. Wells were blocked for 2 h at RT with PBS/0.5% BSA, followed by washing (0.9% NaCl/0.1% Tween-20). Freshly thawed supernatants of the cell cultures and recombinant human cytokine-standards were incubated in duplicates for 2 h at RT in the presence of a biotinylated second anti-cytokine detection antibody. After washing, wells were incubated with HRP-labelled poly-streptavidin (CLB) for 30 min at RT. HRP revelation was performed with 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase (KPL). Colour development was stopped by adding equal volume of 1 M H_2SO_4. Optical density was measured at 450 nm.

CCL18 levels were measured by sandwich ELISA assay using a commercially available CytoSet (Biosource), consisting of a capture-antibody, a biotinylated detection antibody, recombinant CCL18 standard and streptavidin-HRP conjugate. Assay conditions were exactly as described by the manufacturer.

Real-time quantitative PCR

To quantify mRNA expression by foamy macrophages total RNA was extracted from cell cultures using the GenElute Mammalian Total RNA kit (Sigma). RNA samples were treated with DNAse I (Invitrogen) to remove any contaminating DNA. Using 1 μg of the total RNA as template, copy DNA (cDNA) was prepared using the AMV Reverse Transcription System (Promega). To determine target gene mRNA expression, real-time quantitative reverse-transcription-PCR was performed using TaqMan technology (PE-Applied Biosystems) as described previously (van der Fits *et al.*, 2003). Target gene expression levels were corrected for GAPDH mRNA levels. Sequences of the PCR primers (PE Biosystems) and fluorogenic probes (Eurogentec or Roche) are: forward primer 5′-CCTTCCTCCTGTGCCTGATG, reverse primer 5′-ACAATCTCATTTGAATCAGGAA, probe 5′-TGCCCGACTCCCTTGGGTGTCA for COX-2; forward primer 5′-ACGGCGCTGTCATCGATT, reverse primer 5′-GGCATTCTTCACCTGCTCCA, probe 5′-CTTCCCTGTGAAAACAAGAGCAAGGCC (Eurogentec) for IL-10; forward primer 5′-GCCCAGGCAGTCAGATCATC, reverse primer 5′-GGGTTTGCTACAACATGGGCT, probe 5′-CTCGAACCCCGAGTGACAAGCCTG (Eurogentec) for TNF-α; forward primer 5′-CACCGGAACGACATGGAGA, reverse primer 5′-TCCAGGCGACAAAAGGGTTA, probe 5′-TGGGCTTCGTCTACTCCTTTCTGGGTC (Eurogentec) for PGES; forward primer 5′GCCTGGCCTCCAGAAAGACC, reverse primer 5′-ACCTGGTACATCTTCAAGTCTTCATAAAT, probe 5′-CTTTTATGATGGCCCTGTGCCTTAGT (Eurogentec) for IL-12p35; forward primer 5′-GTTCCCCATATCCAGTGTGG, reverse primer 5′-TCCTTTGCAAGCAGAACTGA, probe TGGCTGTG (Roche) for IL-23p19; forward primer 5′-GCCATATGGGAACTGAAGAAA, reverse primer 5′-GGGGTGTCACAGGTGAGG, probe CCTGGAGA (Roche) for IL-12/23p40; forward primer 5′-GCCAGGAGTTGTGAGTTTCCA, reverse primer 5′-TGCAAGGCCCTTCATGATG, probe 5′-TCTGACCACTTCTCTGCCTGCCCA for CCL18.

Statistical analysis

Statistical analysis was performed using the non-parametric Mann–Whitney analysis. P-values < 0.05 were considered significant.

Results

Foamy macrophages express anti-inflammatory markers and demonstrate a unique location-dependent phenotype

To determine the immune phenotype of lipid-laden foamy macrophages in multiple sclerosis lesions, we used antibodies against CNS proteins, various surface markers involved in antigen recognition and presentation and pro- and anti-inflammatory markers characteristic for M1 and M2 macrophages (Kodelja *et al.*, 1998; Goerdt *et al.*, 1999; Mantovani *et al.*, 2002; Gordon, 2003). Figure 1 shows a typical multiple

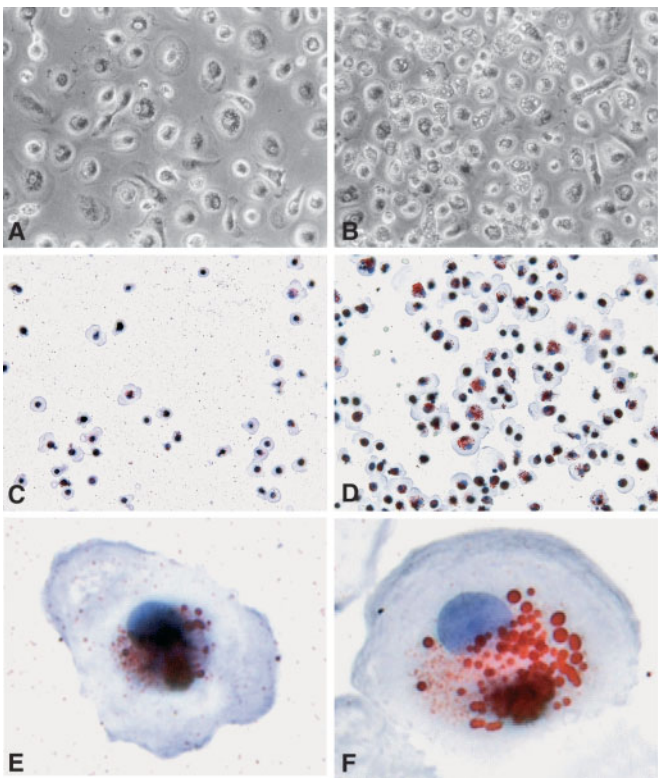
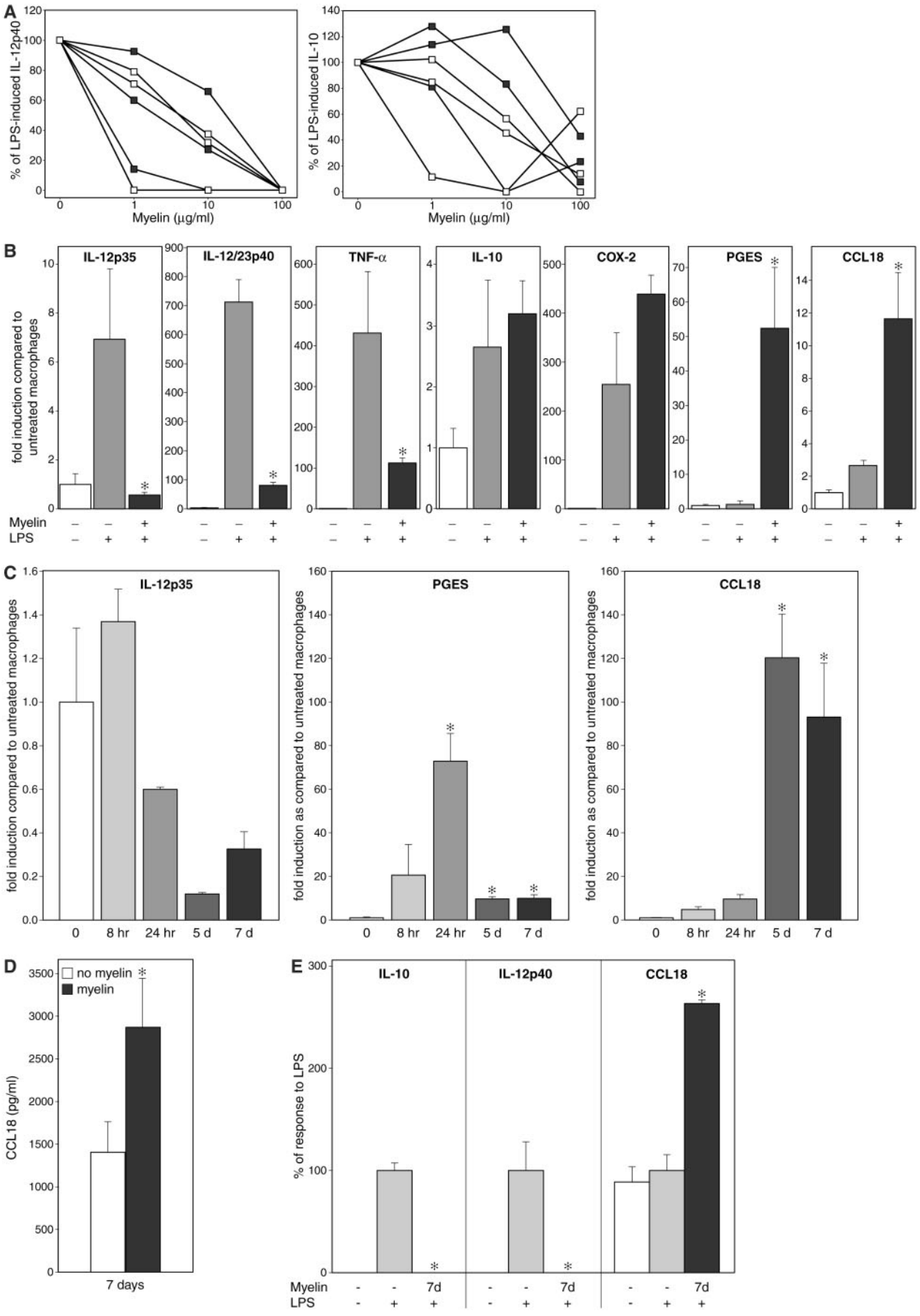


Fig. 4 Human *in vitro* model for myelin-driven foamy macrophage formation. Human monocyte-derived macrophages were cultured in the absence (**A, C, E**) or presence (**B, E, F**) of human brain-derived myelin for 24 h. Whereas cells cultured in the absence of myelin did not appear foamy (**A**, magnification ×32), those cultured with myelin acquired a characteristic foamy morphology as observed by light microscopy (**B**, magnification ×32). (**C**) Fewer macrophages contained neutral lipid-containing vesicles compared to (**D**) myelin-laden macrophages. (**E**) Macrophages contained smaller and fewer neutral lipid-containing vesicles than (**F**) myelin-laden macrophages. **E** and **F** are enlargements of **C** and **D**, respectively.



sclerosis lesion with a clear loss of myelin as demonstrated by myelin oligodendrocyte protein (MOG) staining (Fig. 1A). Foamy macrophages were defined by their characteristic morphology, strong HLA-DR expression and the presence of neutral lipids, which are detected by oil-red O histochemistry (ORO, Fig. 1B and C). To determine whether foamy macrophages display phenotypic and functional specialization dependent on micro-location, we analysed the phenotype of these cells in different micro-locations (Fig. 1D). Thus, we distinguished between foamy macrophages within the lesion, in perivascular spaces within the lesion and in the outer or inner rim (Fig. 1D). The distinction between the outer and inner rim was based on the presence of neutral lipids, MOG and on the size of the foamy macrophages. Outer rim foamy macrophages were smaller in size and contained more MOG, but less neutral lipids than inner rim foamy macrophages.

IL-6, a cytokine with pro- as well as anti-inflammatory properties as well as the anti-inflammatory M2 marker IL-1ra and prostaglandin E_2 synthase (PGES) were differentially expressed in the distinct areas of the lesion (Fig. 2A–C). Whereas IL-6 and IL-1ra were detected mostly in perivascular and lesional foamy macrophages, PGES was mostly expressed in the outer and to a lesser extent in the inner rim. Importantly, expression patterns between cells varied even when cells were in close proximity. Mannose receptor, which is characteristic for M2 macrophages (Mantovani *et al.*, 2002, 2004; Gordon, 2003; Mosser, 2003), was highly expressed on foamy macrophages in perivascular spaces (Fig. 2D, arrows) but was mostly absent on parenchymal foamy macrophages (Fig. 2D, arrowheads). Occasionally, a weakly positive cell was observed (Fig. 2D, asterisk) which was always in the vicinity of a blood vessel. TGF-β expression showed the reverse expression pattern with more pronounced expression by parenchymal foamy macrophages compared to perivascular foamy macrophages (Fig. 2E, arrowheads and arrows, respectively).

As hypothesized, the relative levels of expression were related to specific micro-locations within the lesion (Fig. 3). Foamy macrophages in the lesion rim contained MOG and immunoreactivity showed a decreasing trend towards the centre of the lesion, possibly reflecting time-dependent myelin degradation. In contrast, intracellular neuronal antigen MAP-2 immunoreactivity increased towards the centre of the lesion, implicating that neuronal damage occurs mostly in the lesion centre. Only foamy macrophages within perivascular spaces expressed the surface markers CD11b, CD163 and mannose receptor (Fig. 3). The anti-inflammatory molecules IL-1ra, CCL18, IL-10, TGF-β and IL-4 were all expressed by foamy macrophages and expression was highest in the centre of the lesion (Fig. 3). Interestingly, IL-10 expression was not found in foamy macrophages in perivascular spaces. The pro-inflammatory cytokines TNF-α, IL-1β, IL-12p40/70 were not expressed by foamy macrophages in any of the micro-locations (Fig. 3), whereas cells associated with vessels in normal appearing white matter (NAWM) did express these pro-inflammatory cytokines. Phenotypic heterogeneity was not observed among non-foamy macrophages which were present in low numbers in perivascular spaces in NAWM. These non-foamy macrophages all expressed markers such as CD11b and CD68 and did not produce the typical anti-inflammatory molecules as observed for the foamy macrophages.

Thus, we demonstrate that foamy macrophages in the brain have clear anti-inflammatory characteristics, resemble M2 macrophages and have a unique phenotype depending on the micro-location.

Myelin induces a foamy morphology in macrophages resembling that of foamy macrophages *in situ*

Next, we set out to determine whether ingestion of myelin *in vitro* results in an anti-inflammatory function of foamy macrophages as observed *in situ*. Therefore, we first developed a fully human *in vitro* model of foamy macrophages. Human primary macrophages obtained from healthy donors were fed with 50 μg/ml human myelin and changes in the morphology were monitored by light microscopy and by ORO staining to detect intracellular neutral lipids. Although small individual changes in kinetics between individual donors were observed, macrophages acquired a foamy morphology between 24 and 48 h (Fig. 4A and B) and contained a markedly increased number and size of lipid droplets in

Fig. 5 Foamy macrophages *in vitro* are immunosuppressive. (**A**) Foamy macrophages were generated using myelin preparations derived from brain tissue of three control individuals and three multiple sclerosis patients. After addition of 1 ng/ml LPS for 24 h cytokine levels in the supernatants were determined. LPS-induced IL-12p40 and IL-10 production was dose-dependently inhibited by myelin and is shown as the percentage of production by untreated LPS-stimulated macrophages. Control patient-derived myelin, filled squares; multiple sclerosis patient-derived myelin, open squares. (**B**) Foamy macrophages were incubated with 50 μg/ml myelin for 24 h and subsequently stimulated with 1 ng/ml LPS for 2 h where indicated. LPS-induced IL-12p35, IL-12/23p40 and TNF-α mRNA levels were significantly inhibited in foamy macrophages compared to control macrophages. LPS-induced IL-10 and COX-2 were not affected. PGES and CCL18 mRNA expression was not induced significantly by LPS and was increased by myelin. *, $P < 0.05$ compared with LPS-treated macrophages. (**C**) Over time, foamy macrophages showed a decreased, but not significant, IL-12p35 mRNA expression. In addition there was a transient increase in PGES and a sustained increase in CCL18 mRNA expression. *, $P < 0.05$ compared with untreated control macrophages. (**D**) This was paralleled on protein level as 7 days after myelin addition foamy macrophages still showed significantly increased CCL18 production. *, $P < 0.05$ compared with untreated control macrophages. (**E**) 7 days after myelin ingestion foamy macrophages showed a complete inhibition of LPS-induced IL-10 and IL-12p40 production and a 3-fold induction of CCL18 production. *, $P < 0.05$ compared with LPS-treated macrophages. All data shown are representative for at least two independent experiments using different blood donors. Results are expressed as mean ± SD.

comparison to control macrophages (i.e. not fed with myelin) as demonstrated by ORO staining (Fig. 4C–F). The typical foamy morphology of macrophages could still be observed 1 week after the initial addition of myelin. Macrophage viability was not affected by myelin ingestion when a dose range of 1–100 μg/ml was used, as demonstrated by trypan blue staining (data not shown).

Foamy macrophages do not mount pro-inflammatory responses to prototypical inflammatory stimuli and produce anti-inflammatory mediators

To assess the effect of myelin ingestion on macrophage function, cytokine levels were determined in supernatants of myelin-laden macrophages before and after LPS stimulation. Since variation in myelin lipid composition between multiple sclerosis and normal brain has been reported (Woelk and Borri, 1973), myelin was isolated from white matter of three control (open symbols) brains and three multiple sclerosis (filled symbols) brains to investigate possible functional differences. Macrophages were incubated with the distinct myelin preparations for 24 h and IL-10 and IL-12p40 levels were determined in the supernatants by ELISA. None of the myelin preparations induced IL-12p40 and only the highest dose of one multiple sclerosis brain-derived myelin was associated with a transient IL-10 induction (data not shown). All myelin preparations inhibited LPS-induced IL-12p40 and IL-10 induction in a dose-dependent fashion (Fig. 5A). No significant differences were observed in cytokine production between foamy macrophages generated using the different myelin preparations. For subsequent experiments 50 μg/ml myelin was used.

Next, the effect of myelin ingestion on LPS-induced mRNA levels of different pro-and anti-inflammatory mediators was determined. Macrophages were incubated with myelin for 24 h and subsequently stimulated with LPS for an additional 2 h, after which RNA was isolated and real-time RT-PCR was performed for IL-12p35, IL-12/23p40, IL-23p19, TNF-α, IL-10, COX-2, PGES and CCL18 (Fig. 5B). LPS-induced IL-12p35, IL-12/23p40 and TNF-α expression by foamy macrophages was dramatically inhibited. LPS-induced IL-23p19 expression was decreased, albeit not significantly (data not shown). IL-10 mRNA was slightly but not significantly induced by LPS in control macrophages as well as foamy macrophages. COX-2 was increased after LPS stimulation in control macrophages but this induction was not significantly inhibited in foamy macrophages. Foamy macrophages showed between 15–50 and 8–12-fold induction of CCL18 and PGES compared to control macrophages. Thus, myelin ingestion resulted in a differential modulation of LPS responses. LPS-induced IL-12p35, IL-12/23p40 and TNF-α mRNA expression was strongly and significantly inhibited, IL-23p19, IL-10 and COX-2 expression remained unaffected and the expression of anti-inflammatory CCL18 and PGES significantly increased.

To determine whether myelin ingestion results in long-term modulation of macrophage function, macrophages were incubated with myelin for the indicated time periods and real-time RT-PCR was performed for IL-12p35, IL-10, PGES and CCL18. IL-10 mRNA was not detectable at any time point (data not shown). After myelin uptake IL-12p35 expression was decreased, albeit not significantly, over time in comparison to control macrophages (Fig. 5C). In contrast to IL-12p35 both PGES and CCL18 were induced by myelin (Fig. 5C). Seven-day-old foamy macrophages expressed 10- and 90-fold more PGES and CCL18 than control macrophages. IL-12p40, IL-10 and CCL18 levels were subsequently determined in supernatants of these foamy macrophages. CCL18 is constitutively produced by macrophages and production by foamy macrophages is increased at day 7 after myelin ingestion (Fig. 5D), paralleling the increased CCL18 mRNA expression by foamy macrophages. IL-12p40 and IL-10 were not detectable (data not shown).

Subsequently we determined whether the aberrant LPS response persisted over time. Seven days after initial myelin ingestion foamy macrophages were stimulated with 1 ng/ml LPS for 24 h and cytokine levels in the supernatant were determined by ELISA. LPS-induced IL-12p40 and IL-10 production by these foamy macrophages was abolished completely (Fig. 5E) whereas CCL18 was significantly increased (Fig. 5E). In addition, responses to other prototypical pro-inflammatory stimuli such as peptidoglycan and zymosan were also completely abolished (data not shown).

Discussion

The relapsing-remitting nature of multiple sclerosis strongly suggests the presence of potent counter-regulatory mechanisms that keep the disease in check. One such mechanism may be the active control of inflammation in the CNS itself thus preventing infinite expansion of the demyelinating lesion. Inflammation and demyelination are responsible for at least short-term neurological symptoms. Inflammation probably contributes to axonal loss as neurons are more vulnerable to environmental insults when the protective myelin sheaths are destroyed and the axons exposed (Kuhlmann *et al.*, 2002; Grigoriadis *et al.*, 2004). It is therefore imperative that in the developing lesions the production of toxic molecules is halted and that inflammation is limited allowing for tissue repair (Sospedra and Martin, 2005). Myelin-laden foamy macrophages are abundantly present in demyelinating lesions and although it is generally assumed that these cells contribute to inflammation, evidence for this is scarce (van der Laan *et al.*, 1996). This lack of data on foamy macrophage function in multiple sclerosis is in sharp contrast with the increasing attention for foam cells in atherosclerosis (Greaves and Gordon, 2005) reporting potent immune-regulatory functions by lipids and lipid-induced molecules (Harris *et al.*, 2002; Lawrence *et al.*, 2002; Pettus *et al.*, 2002; Joseph *et al.*, 2003, 2004). Lipid-laden cells are anti-inflammatory (Lawrence *et al.*, 2002) and it was shown that low-density

lipoprotein (LDL) uptake by macrophages inhibits TNF-induced TNF expression and induces IL-10 (Lo *et al.*, 1999; Varadhachary *et al.*, 2001; Ares *et al.*, 2002). Foamy macrophages in the rim of active demyelinating multiple sclerosis lesions have been shown to contain plasma LDL (Newcombe *et al.*, 1994).

Here, we establish that foamy macrophages in active multiple sclerosis lesions have consistent immunosuppressive function, while displaying a unique surface phenotype dependent on the micro-location. In addition, we demonstrate that ingestion of human myelin alters human macrophage function *in vitro* by inducing anti-inflammatory molecules and by inhibiting responses to pro-inflammatory stimuli. The results presented here reveal a new regulatory immunosuppressive pathway in multiple sclerosis.

We demonstrate that foamy macrophages in demyelinating lesions in multiple sclerosis brain express various markers that are involved in anti-inflammatory processes, including IL-1ra, IL-10 CCL18, TGF-β and that a subset of the foamy macrophages express markers involved in innate immunity, including mannose receptor and CD163. These molecules are all characteristic for alternatively activated M2 macrophages (Mantovani *et al.*, 2002; Gordon, 2003; Mosser, 2003) and this strongly suggests a local regulatory immunosuppressive role.

Importantly, our data show that foamy macrophages occur in discrete subsets. This may reflect their origin (i.e. microglial-derived versus blood-derived) and the degree of lipid degradation. Most likely the cues received from their microenvironment are critical. These cues include the type of ingested lipids, cytokine environment, presence and identity of neighbouring cells and signals from the extracellular matrix. The unique combination of surface and intracellular molecules of individual macrophages in different areas of the lesion suggests that they are likely to exert diverse functions depending on their location. Foamy MOG macrophages in the lesion rim may be more involved in phagocytosis of myelin whereas foamy macrophages inside the lesion appear to be geared for downregulation of inflammation as suggested by high expression of anti-inflammatory cytokines. Interestingly, our *in vitro* data show a transient increase in PGES expression and a sustained increase in CCL18 expression. This parallels the *in situ* analysis showing highest expression of PGES in foamy macrophages in the lesion rim that likely have ingested myelin more recently than foamy CCL18-positive macrophages in the lesion centre. Although LPS-induced IL-23p19 expression was not significantly decreased in foamy macrophages, expression of the other subunit of IL-23, p40, was strongly inhibited. This indicates a reduced capacity of foamy macrophages to produce both IL-12 as IL-23 after LPS stimulation. Importantly, the p40 subunit is critical in the development of EAE, an animal model for multiple sclerosis and forms the basis of the Th1 bias of the CNS (Becher *et al.*, 2003). IL-10 was expressed *in situ* mostly by lesional foamy macrophages. However, in *in vitro* LPS-induced IL-10 production is strongly inhibited upon myelin ingestion. This suggests complex regulation of IL-10 expression both *in vitro* and *in vivo* warranting more detailed exploration. Regulatory foamy macrophages in perivascular spaces are likely to affect the function of newly infiltrating cells. Current experiments employ genomic as well biochemical approaches to identify such immunomodulating mechanisms.

We hypothesize that the observed functional phenotype of foamy macrophages in multiple sclerosis lesions results from the accumulation of lipids derived from myelin and phagocytosed apoptotic cell membranes, in concert with local micro-environmental cues, such as differences in extracellular matrix content in the perivascular infiltrate versus the lesion in the brain parenchyma. Foamy macrophages demonstrate a phenotype resembling that of anti-inflammatory M2 macrophages, are likely to contribute to resolution of inflammation and may therefore be responsible for inhibiting further lesion development and promoting lesion repair. In addition, they may also function as a first line of defence against infiltrating inflammatory myeloid cells. Future studies are required to elucidate which lipid components are able to regulate macrophage function and which mechanisms are involved. Understanding the mechanisms behind naturally occurring counter-regulatory processes will allow for definition of new cellular targets for therapeutic drug design for the treatment of multiple sclerosis and may well have broader applications for other foam cell-associated diseases including atherosclerosis.

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