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Protective autoimmunity: interferon-γ enables microglia to remove glutamate without evoking inflammatory mediators

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Abstract

Glutamate in excessive amounts is a major contributor to neuronal degeneration, and its removal is attributed mainly to astrocytes. Traumatic injury to the central nervous system (CNS) is often accompanied by disappearance of astrocytes from the lesion site and failure of the remaining cells to withstand the ensuing toxicity. Microglia that repopulate the lesion site are the usual suspects for causing redox imbalance and inflammation and thus further exacerbating the neurotoxicity. However, our group recently demonstrated that early post-injury activation of microglia as antigen-presenting cells correlates with an ability to withstand injurious conditions. Moreover, we found that T cells reactive to CNS-specific self-antigens protected neurons against glutamate toxicity. Here, we show that antigen-specific autoimmune T cells, by tailoring the microglial phenotype, can increase the ability of microglia-enriched cultures to remove glutamate. This T-cell-mediated effect could not be achieved by the potent microglia-activating agent lipopolysaccharide (LPS), but was dose-dependently reproduced by the Th1 cytokine interferon (IFN)-γ and significantly reduced by neutralizing anti-IFN-γ antibodies. Under the same conditions, IFN-γ had no effect on cultured astrocytes. Up-regulation of glutamate uptake induced by IFN-γ activation was not accompanied by the acute inflammatory response seen in LPS-activated cultures. These findings suggest that T cells or their cytokines can cause microglia to adopt a phenotype that facilitates rather than impairs glutamate clearance, possibly contributing to restoration of homeostasis.

Keywords: central nervous system degeneration, central nervous system inflammation, glutamate toxicity, neuroprotection, protective autoimmunity.
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Glutamate, a major excitatory amino acid in the CNS, can turn neurotoxic under various pathological conditions, including acute CNS trauma such as brain or axonal injury (Alessandri and Bullock 1998), ischaemia (Lipton 1999) and epilepsy (Fountain 2000), as well as in chronic neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis and glaucoma (Choi 1988). Clearance of glutamate and maintenance of its homeostasis are attributed largely to astrocytes, acting via high-affinity, sodium-dependent transporters (Danbolt 2001). Glutamate uptake is tightly regulated by the glial expression and post-translational modification of glutamate transporters (Gegelashvili and Schousboe 1998) and probably also by glial response to extracellular glutamate concentrations via metabotrobic glutamate receptors (Aronica *et al.* 2003). Under degenerative conditions, an excess of glutamate is often accompanied by a deficiency in astroglial clearance ability. Adding insult to injury, glutamate becomes locked into a vicious neurotoxic cycle in which excessive extracellular glutamate triggers the formation of free oxygen species and other excitotoxins (Volterra *et al.* 1994a,b), which in turn further impair the structure and function of glutamate trans-

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Abbreviations used: Ab, antibodies; CNS, central nervous system; COX-2, cyclooxygenase-2; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; IFN, interferon; iNOS, induced nitric oxide synthetase; LPS, lipopolysaccharide; NF, nuclear factor; PBS, phosphate-buffered saline; PGE_2, prostglandin E_2; RTPCR, reverse transcription polymerase chain reaction; TNF-α, tumour necrosis factor-α.

porters (Piani *et al.* 1993; Trotti *et al.* 1996; Sorg *et al.* 1997; Muller *et al.* 1998).

Inflammation contributes an additional dimension to the neurotoxic cycle, resulting in further tissue loss through the induction of enzymes such as induced nitric oxide synthase (iNOS), which causes further oxidative imbalance, and the inflammation-mediating enzyme cyclooxygenase-2 (COX-2), known to impair glutamate uptake (Bal-Price and Brown 2001). In addition, end products of the activation of innate immunity, such as prostglandin (PGE_2), NO, and tumour necrosis factor (TNF)-α, although effective in destroying invading micro-organisms, can prove disastrous in the presence of toxic amounts of glutamate. Besides acting directly as neurotoxins, NO and TNF-α can inhibit glutamate transport (Fine *et al.* 1996), and PGE_2 can promote neuronal death by inducing glutamate release from glial cells (Volterra *et al.* 1994a). In light of the bad reputation of inflammation in the CNS, as well as *in-vitro* studies showing that microglia are a major source of TNF-α, NO and PGE_2 (Choi *et al.* 2003), these cells are often viewed as significant contributors to the pathology (Lehnardt *et al.* 2003).

Despite the apparently destructive immune activities outlined above, it is commonly assumed that the aim of an immune response is elimination of a given threat. Clearly, if it is to achieve this goal, it must acquire properties that allow it to rise to the challenge. It was recently suggested that macrophages and microglia might play an active role in glutamate clearance (van Landeghem *et al.* 2001). Human macrophages express functional sodium-dependent glutamate transporters (such as EAAT-1 and EAAT-2) and are able to take up glutamate as efficiently as neurons and astrocytes do (Rimaniol *et al.* 2000). Microglia, by expressing the glutamate transporter GLT-1 (EAAT-2), might act as active glutamate scavengers (Nakajima *et al.* 2001). Moreover, a protective immune response that will eradicate the danger and minimize the loss of host tissue (neurons) must be regulated and shaped by a well-balanced innate-adaptive dialogue, initiated by microglia (part of the innate immune system) and continued and regulated by T lymphocytes (adaptive immune system).

Recent studies in our laboratory, using rodent models of the mechanically injured optic nerve or spinal cord, or the glutamate-intoxicated eye, have demonstrated that the ability to withstand these insults is dependent on a T-cell-mediated response directed against self-antigens residing in the site of stress, and that this response can be boosted by self or self-like antigens (Moalem *et al.* 1999; Hauben *et al.* 2000a,b; Kipnis *et al.* 2000; Hauben *et al.* 2001a,b; Schori *et al.* 2001; Mizrahi *et al.* 2002). We recently demonstrated a correlation between reduction of extracellular hostility of the post-traumatic CNS and early conversion of the microglial phenotype into one that supports antigen presentation by these cells (Shaked *et al.* 2004). Moreover, after passive transfer of T cells in a rodent model of spinal cord injury, the neuroprotective T cells were found to be co-localized in the vicinity of microglia expressing class II major histocompatibility complexes (MHC-II) and the co-stimulatory factor B-7.2 (Butovsky *et al.* 2001). These observations led us to suggest that the beneficial effect of T cells in weakening the harmful effects of the traumatized CNS is a result of their dialogue with resident microglia. Accordingly, we postulated that if microglia are indeed ambivalent in their effects on glutamate homeostasis, i.e. if they are capable both of exacerbating and of reducing glutamate toxicity, their strict regulation (determined, at least in part, by T cells) is of crucial importance to the outcome of CNS injury.

In the present study, using the rat optic nerve as a model, we showed that mature astrocytes disappeared following axonal injury and the lesion site was repopulated by microglia. This finding prompted us to assess two fundamental and seemingly contradictory aspects of microglial activity with respect to glutamate toxicity and T-cell behaviour, namely the removal of extracellular glutamate and the production of pro-inflammatory mediators. We showed that activated T-helper cells (Th) could significantly increase the ability of a microglia-enriched cell culture to scavenge neurotoxic extracellular glutamate. This effect was simulated by interferon (IFN)-γ, a typical Th-1 cytokine, but not by the potent microglial activating agent lipopolysaccharide (LPS). Neutralizing antibodies (Ab) directed against IFN-γ (anti-IFN-γ) significantly reduced the T-cell-mediated effect. Under the same conditions, cultured astrocytes were not affected by IFN-γ. The ability of IFN-γ to promote glutamate uptake was not accompanied by induction of the acute phase of the inflammatory response, which is up-regulated for example by LPS and is likely to obstruct the removal and intensify the toxicity of glutamate.

Materials and methods

Crush injury of the optic nerve

Rats were deeply anaesthetized by injection of XYL-2% (xylazine, 10 mg/kg; Arendonk, Brussels, Belgium) and Ketaset (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA, USA). The optic nerve was exposed under a binocular operating microscope, and calibrated cross-action forceps were used to inflict a crush injury on the nerve 12 mm from the eye. The uninjured contralateral nerve was left undisturbed.

Immunohistochemistry

Longitudinal cryosections of the excised optic nerve (10 μm thick) were picked up onto gelatin-coated glass slides. The sections were fixed in ethanol for 10 min at room temperature, washed twice with double-distilled water and incubated for 3 min in phophate-buffered saline (PBS) containing 0.05% Tween-20. Sections were incubated for 1 h at room temperature with mouse anti-rat monoclonal antibodies (mAb) against MHC-II [mouse anti-rat MHC-II (IHC-306); clone HIS28], mouse anti-rat Ab against CD68, a phagocytic marker (ED-1; clone MCA341R) or mouse mAb against rat glial fibrillary acidic protein (GFAP), an astrocyte marker (clone G-A-5, all from Sigma-Aldrich, Rehovot, Israel), each diluted in PBS

containing 3% fetal calf serum (FCS) and 2% bovine serum albumin. The sections were washed three times with PBS containing 0.05% Tween-20 and incubated with Cyk3-conjugated donkey anti-mouse IgG for 1 h at room temperature. They were then washed with PBS containing Tween-20 and treated with glycerol containing 1,4-diazobicyclo-(2,2,2)-octane to inhibit quenching of fluorescence. Sections were viewed with a Zeiss confocal microscope.

T-cell line

The T-cell line (T_{mbp}) was generated from draining lymph node cells obtained from Lewis rats immunized with myelin basic protein (MBP), as previously described (Ben-Nun and Cohen 1982a,b,c; Moalem *et al.* 2000). The antigen was dissolved in PBS (1 mg/mL) and emulsified with an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) supplemented with 4 mg/mL *Mycobacterium tuberculosis* (Difco). Ten days after the antigen was injected (in 0.1 mL of the emulsion) into the rats' hind footpads, the rats were killed and draining lymph nodes were surgically removed and dissociated. For production of activated T_{mbp} cells, the cells were washed and activated by incubation with their specific antigen, MBP (10 μg/mL), in a proliferation medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), 2-mercaptoethanol (5×10^5 M), sodium pyruvate (1 mM), penicillin (100 IU/mL), streptomycin (100 g/mL), non-essential amino acids and autologous rat serum 1% (volume/volume). After incubation for 72 h at 37C, 90% relative humidity and 7% CO_2, the cells were transferred to a propagation medium consisting of DMEM, L-glutamine, 2-mercaptoethanol, sodium pyruvate, non-essential amino acids and antibiotics, in the same concentrations as above, as well as FCS (10% volume/volume) and 10% T-cell growth factor derived from the supernatant of concanavalin A-stimulated spleen cells. The cells were incubated in the propagation medium for 4 to 10 days, after which they were activated by re-stimulation with MBP (10 μg/mL) in the presence of irradiated thymus cells (10^7 cells/mL; 2000 rad) in proliferation medium. Non-activated T_{mbp} cells were prepared in the same way as the activated cells, but were not exposed to re-stimulation with MBP. The T-cell lines were expanded by repeated stimulation and propagation. The T_{mbp} cells used in this study were previously characterized as T-helper (Th) 0 cells with a Th1 bias (Moalem *et al.* 2000).

Phagocyte isolation and FACS analysis

To isolate cells from the rat spinal cord, Lewis rats (8 weeks old) were deeply anaesthetized as described above and perfused intracardially with PBS. Spinal cords were incubated with collagenase II (1 mg/mL; Sigma), at 37°C for 20 min and mononuclear cells were then isolated by discontinuous Percoll gradient. For FACS analysis, the cells were washed in FACS buffer (1% FCS, 0.1% sodium azide in PBS) and, after blocking with purified goat IgG, were stained with directly conjugated antibodies. For purification of CNS phagocytes, the cells were stained with anti-Mac-1-FITC or anti-CD45-CyChrom or both. Mac-1 + $CD45^{high}$ (macrophage) and Mac-1 + $CD45^{low}$ (microglial) populations were collected by FACStarplus (Becton Dickinson, Franklin Lakes, NJ, USA).

Co-culturing of microglia and T_{mbp} cells

Glial cells (microglia or astrocytes) were isolated from the cerebral cortex of newborn Lewis rats as previously described (Corradin *et al.* 1993; Chan *et al.* 2001). The cells were grown as a mixed glial culture for 7 days. Microglia were separated from other glial cells by shaking (80 r.p.m.) for 30 min at 37°C. The cells were then seeded on 24-well plates (1×10^5 microglia and 2×10^4 astrocytes/mL in each well) coated with poly D-lysine (20 mg/mL; Sigma-Aldrich) and incubated at 37°C, 5% CO_2 for 3 days in RPMI-1640 medium containing 10% FCS, 50 mM β-mercaptoethanol (all from Sigma-Aldrich), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin. T_{mbp}-activated cells were then added. Microglial purity in some of the cultures was assessed immunohistochemically with IB-4 (a marker of activated microglia) (Streit and Kreutzberg 1987), and was found to be 95%. GFAP-immunoreactive cells (astrocytes) in these cultures accounted for less than 1.5% of the population. Astrocyte purity in the cultures, assessed immunohistochemically with GFAP, was not less than 95%. Microglia and T_{mbp}-activated cells (2×10^4 cells/mL/well) were co-cultured for 24 h in the presence or absence of anti-IFN-γ (2.5 μg/mL). The microglia were then washed three times with PBS and prepared for immunocytochemistry. In a separate experiment, microglia and T_{mbp} cells were co-cultured in a transwell using BD Falcon™ cell culture inserts, pore size 0.4 μm (Becton Dickinson, Le Pont De Claix, France).

Neutralization of IFN-γ

To neutralize secreted IFN-γ, we added anti-rat IFN-γ antibody (anti-IFN-γ Ab; R & D Systems, Minneapolis, MN, USA; catalogue no. AF-585-NA) to enriched microglial cultures treated with activated T_{mbp} cells.

Glutamate uptake

Uptake of glutamate was measured essentially according to the method of Swanson *et al.* (1997). Briefly, the medium in the wells was replaced with 0.5 mL of Hanks's balanced salt solution (HBSS) and maintained for 30 min at 37°C in 10% CO_2. The contents of each well were then incubated at 37°C with 0.5 mL of reaction mixture (1–200 μM/0.05 Ci (L-[U]-^{14}C glutamate/HBSS) for 10 to 60 min. Each well was then rinsed twice with cold HBSS and its contents were solubilized with 200 ml of lysis buffer (0.5 N NaOH/0.05% sodium dodecyl sulfate). ^{14}C-glutamate was determined in 100-ml aliquots using a scintillation counter. Protein content was determined using the Pierce BCA protein assay kit (Pierce, Rockford, IL, USA). In experiments aimed at examining the dependence of glutamate uptake on sodium, sodium was replaced with choline chloride (137 mM).

Determination of NO_2

Release of NO_2 was assayed according to the method of Griess (Green *et al.* 1982). Briefly, equal volumes of Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H_3PO_4) were incubated with supernatant samples (100 μL of medium in which microglia had been cultured) for 10–20 min at room temperature and absorbance was measured at 550 nm in a micro-ELISA reader with a reference filter of 620 nm. The NO_2 concentration (in μM) was determined using $NaNO_2$ as a standard.

Reverse-transcription PCR

We assayed the expression of specific mRNAs by reverse-transcription PCR (RTPCR), using the selected gene-specific primer pairs listed in Table 1. RNA was reverse-transcribed with SuperScript II, as recommended by the manufacturer. RTPCR reactions were carried out in 30-μL reactions mixtures containing 1 μg of cDNA, 35 nmol of each primer and ReadyMix PCR Master Mix (ABgene, Epsom, UK). The PCR products were assessed using 1.5% gel agarose.

Quantitative reverse-transcription PCR

We assayed specific mRNA levels by quantitative RTPCR (Q-RTPCR), using the selected gene-specific primer pairs listed in Table 2. RNA was reverse-transcribed with SuperScript II and random primers, as suggested by the manufacturer. PCR reactions were carried out using 10 ng cDNA, 50 nM of each primer and SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in 10-ml reactions. The Q-RTPCR products were assessed by measuring the SYBR green fluorescence (Ririe *et al.* 1997; Wittwer *et al.* 1997) collected during real-time PCR on an ABI Prism 7900HT sequence detection system (Applied Biosystems). A control cDNA dilution series was created for each gene to establish a standard curve. Each reaction was subjected to melting-point analysis to confirm single amplified products. The primers used are shown in Table 2.

Microarrays

Samples of RNA from cultured microglia were assayed on spotted rat oligonucleotide microarrays as described previously (Carmel *et al.* 2001). Briefly, 4967 oligonucleotides, each consisting of 6570 nucleotides, were purchased from Compugen (Jamesburg, NJ, USA) or MWG Biotech (Ebersberg, Germany) and spotted on poly D-lysine-coated glass slides. Probes were prepared with the Genisphere (Hatfield, PA, USA) 3-DNA labelling system. Hybridizations were performed on a Discovery Workstation (Ventana Medical Systems, Tuscon, AZ, USA). Images were collected on an Axon GenePix 4000B laser scanner (Axon Instruments, Union City, CA, USA), Lowess-normalized (Yang *et al.* 2002) and loaded into GeneSpring (Silicon Genetics, Redwood City, CA, USA) for analysis.

Results

Microglia repopulate the injured optic nerve

We first sought to identify the cellular elements that repopulate the lesion site after intra-orbital crush of the rat optic nerve. To delineate the lesion site, we used antibodies against the astrocyte marker GFAP (Blaugrund *et al.* 1993). Within the site demarcated by anti-GFAP antibodies (Fig. 1a), GFAP immunoreactivity was absent. The injury site was populated instead by activated microglia, identified by their positive staining both with ED1 antibodies (CD68, a glycosylated lysosomal antigen expressed on activated phagocytes; Fig. 1b) and with antibodies against MHC-II molecules (Fig. 1c). It should be emphasized that the activated phagocytes (ED1+ cells) could be both CNS-resident microglia and blood-borne macrophages.

It was recently suggested that microglia and macrophages can be distinguished by their different levels of expression of Mac-1 (CD11b; an α-chain of the CD11b/CD18 glycoprotein complex) and CD45, a common leukocyte antigen (LCA): whereas infiltrating macrophages are Mac-1+/CD45^{high}, resident microglia are Mac-1+/CD45^{low} (Juedes *et al.* 2000). To determine the relative amounts of microglia and macrophages, we subjected phagocytes isolated from injured rat spinal cords to FACS analysis. At the time points examined (18 h, 3 days and 8 days after injury), most of the Mac-1+ cells were CD45^{low}, indicative of activated microglia. The relative amount of CD45^{low} cells (activated microglia) at the injury site increased up to day 3 and then stabilized (the numbers of Mac-1+/CD45^{low} cells, expressed as a percentage of total extracted cells, were 36.44, 56.7 and 55.23%, respectively, at the three time points), whereas the corresponding relative amounts of CD45^{high} cells (blood-borne macrophages) were 0, 3.02 and 7.79%).

These results, taken together with previous results from our laboratory demonstrating that in rats subjected to passive or active T-cell-based immunization, optic nerve crush injury or spinal cord contusion was followed by co-localization of T cells and microglia (Butovsky *et al.* 2001; Shaked *et al.* 2004), prompted us to carry out an *in-vitro* examination of features that are conferred on the microglia as a result of their dialogue with the T cells. The T_{mbp} cells used for passive immunization after optic nerve crush injury or spinal cord contusion were characterized as Th0 cells with a Th1 bias (Moalem *et al.* 2000).

Table 1. RT-PCR primer sequences

Gene	Accession		Sequence	Product length	PCR cycles
iNOS	U03699	F	5'- TGAGACAGGAAAGTCGGAAG -3'	480	27
		R	5'- TCCCATGTTGCGTTGGAA -3'		
IκBα	XM_343065	F	5'- CATGAAGAGAAGACACTGACCATGGAA-3'	328	26
		R	5'- TGGATAGAGGCTAAGTGTAGACACG-3'		
COX-2	AF233596	F	5'- ACTTGCTCACTTGTTGAGTCATT-3'	547	30
		R	5'- TTTGATTAGTACTGTAGGGTTAAT-3'		
L-19	M62952	F	5'- CTGAAGGTCAAAGGGAATGTG -3'	197	20
		R	5'- GGACAGAGTCTTGATGATCTC -3'		

Table 2. Q-RT-PCR primer sequences.

Gene	Acceession		Sequence
IL-6	NM_012589	F	5'-ATTCTGTCTCGAGCCCACCA-3'
		R	5'-CTGAAGGGCAGATGGAGTTGA-3'
COX-2	NM_017232	F	5'-GAGTGGGATGACGAGCGACT-3'
		R	5'-TCCTATCAGTATGAGCCTGCTGG-3'
CD-14	NM_021744	F	5'-CTTTGACACTGGTGCATGCC-3'
		R	5'-CCAGCTCACAGGGTTCTGGT-3'
iNOS	NM_012611	F	5'-TGGAGCATCCCAAGTACGAGT-3'
		R	5'-CATACCACTTCAGCCCGAGC-3'
TLR4	NM_019178	F	5'-CTGGGTTTCTGCTGTGGACA-3'
		R	5'-AGGTTAGAAGCCTCGTGCTCC-3'
IκBα	XM_343065	F	5'-CTCACGGAGGACGGAGACTC-3'
		R	5'-CTCTTCGTGGATGATTGCCA-3'
STAT-1	AF205604	F	5'-AACGGTCCCAAAATGGAGGT-3'
		R	5'-TGTAGGGCTCAACAGCATGG-3'

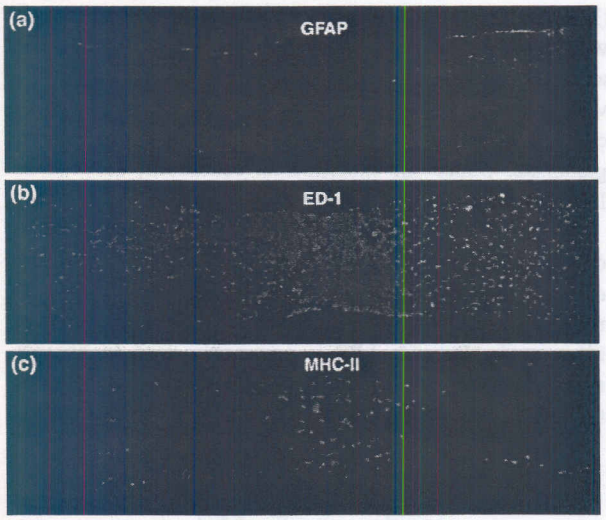


Fig. 1 Microglia/macrophages repopulate the site of CNS injury. The figure shows expression of GFAP [a marker of astrocytes (a)], ED1 [CD68, a marker of phagocytes (b)] and MHC-II [a marker of antigen presentation (c)] after intraorbital crush of the optic nerve in Lewis rats. CD68 and MHC-II are both characteristic of microglia. Two weeks after injury, longitudinal cryosections were stained for GFAP, ED1 and MHC-II. Note the absence of astrocytes at the lesion site and the accumulation of microglia/macrophages.

Activated T_{mbp} cells improve microglial capacity for glutamate uptake

We cultured an enriched population of microglial cells (1×10^5 cells per well) (Corradin *et al.* 1993; Chan *et al.* 2001). Microglial purity was examined in some of the preparations and was found to be 95%. In such cultures, GFAP-immunoreactive cells (astrocytes) accounted for less than 1.5% of the population. The capacity of the microglia for glutamate uptake was assayed as a function of the number of co-cultured activated T_{mbp} cells (5×10^3 to 1×10^5). As the optimal number of activated T_{mbp} cells for inducing glutamate uptake by the microglia was found to be 2×10^4 (data not shown), this was the number of T_{mbp} cells (activated by re-stimulation with their antigen (Moalem *et al.* 2000); see Materials and methods) that we chose to co-incubate with the enriched microglial preparations.

The ability of the co-incubated microglia to clear the medium of [^{14}C]-glutamate at the stress-related concentration of 40 μM (van Landeghem *et al.* 2001) was assayed and compared with that of control preparations of enriched microglia that were cultured in the absence of T cells. Incubation of the microglia in medium containing [^{14}C]-glutamate (40 μM) for 30 min (see Materials and methods) showed that, within the time frame of our experiments, the treatment had no detectable effect on cell viability. Figure 2(a) shows that the ability of the microglia to take up glutamate was significantly up-regulated by the activated T_{mbp} cells. In the absence of sodium (i.e. when 137 mM NaCl in the medium was replaced with 137 mM choline chloride) the glutamate-uptake ability of both T_{mbp}-treated and -untreated microglia was almost completely blocked, in line with reports that glutamate uptake is most commonly mediated via high-affinity sodium-dependent transporters (Billups *et al.* 1996; Zerangue and Kavanaugh 1996; Levy *et al.* 1998). Both basal and T-cell-induced uptake of glutamate was wiped out upon addition of the competitive transportable EAAT1-4 inhibitor, L-(−)-threo-3-hydroxyaspartic acid (data not shown). Figure 2(b) shows that activated T_{mbp} cells were significantly more potent than non-activated T_{mbp} cells in up-regulating the glutamate-uptake capacity of microglia. Prior to glutamate assay, the microglial cultures were thoroughly washed to remove T cells. To eliminate the possibility that even traces of T cells might have contributed to the observed increase in glutamate uptake, we measured the uptake of glutamate by cultured T cells. The modest glutamate-uptake ability of T cells was found to be sodium independent, ruling out their possible contribution to the measured uptake.

In general, antigenic specificity of Th cells is known to be needed for their homing and activation, enabling them to perform immunological tasks. Once activated, their activity (via soluble substances such as cytokines) is no longer antigen dependent. To determine whether the observed ability of activated T_{mbp} cells to induce glutamate uptake is indeed mediated by T-cell-derived soluble factor(s), we co-cultured microglia with activated T_{mbp} cells using a Falcon transwell filter (pore size 0.4 μm) that allows the passage of soluble substances from the activated T_{mbp} cells to the microglia, even when there is no direct contact between the two cell populations. Figure 2(c) shows that under these conditions activated T_{mbp} cells, although deprived of any cell–cell interaction, nevertheless induced microglial

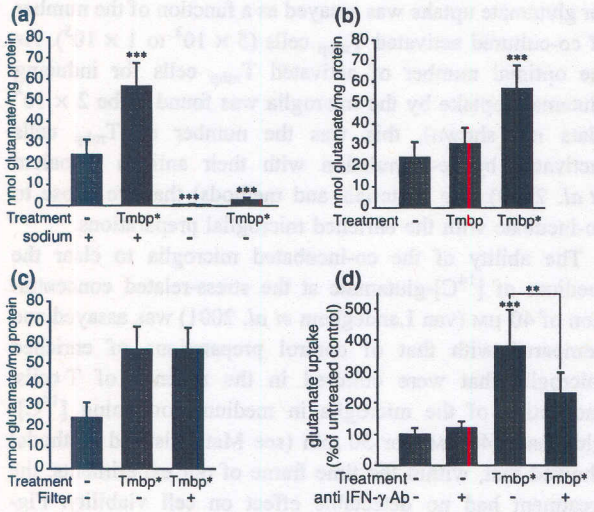


Fig. 2 MBP-activated T cells enhance the ability of microglia-enriched cultures to take up extracellular glutamate at stress-related concentrations. Cultures enriched in rat microglia were co-cultured for 24 h with activated T_{mbp} cells (T_{mbp}*) and were then incubated for 30 min at 37°C in uptake medium (see Materials and methods) in the presence (137 mM NaCl) or absence of sodium (with 137 mM choline chloride substituted for NaCl) and with glutamate at the stress-related concentration of 40 μM (40 μM/0.05 Ci L-[u]^{14}C-glutamate) (a), or cultured with non-activated (T_{mbp}) or activated (T_{mbp}*) (b), or with activated T_{mbp} cells while preventing or allowing cell/cell interactions using transwell filters (c). Glutamate concentrations were assayed as described in Materials and methods and are expressed as nmol glutamate/mg protein. The uptake of glutamate induced by activated T_{mbp} cells could be inhibited by the addition of anti-IFN-γ Ab, suggesting that the T-cell effect is mediated, at least in part, via IFN-γ (d). Values are means ± SD of three separate experiments, each carried out with eight replicates.

up-regulation of glutamate uptake. These results appeared to confirm that the effect of T cells on the ability of microglia to take up glutamate from the extracellular environment is mediated by soluble factors (such as cytokines), known to increase in amount after T-cell activation.

IFN-γ is a characteristic cytokine of Th1 cells, which manifest a protective phenotype (Kipnis *et al.* 2002). We therefore examined whether the effect of activated T_{mbp} cells on glutamate uptake is mediated via IFN-γ. We did this by adding anti-IFN-γ Ab to enriched microglial cultures that were treated with activated or non-activated T_{mbp} cells or were untreated. Figure 2(d) shows that addition of these antibodies (2.5 μg/mL) significantly reduced (by 40%) the ability of activated T_{mbp} cells to induce microglial up-regulation of glutamate uptake, but did not affect glutamate uptake by microglia in the absence of T cells. Addition of anti-IFN-γ Ab to untreated microglia had no effect, indicating that these antibodies by themselves had no effect on glutamate uptake (not shown).

The finding that up-regulation of glutamate uptake is induced by activated T_{mbp} cells prompted us to examine whether such T cells can enable microglia to maintain their capacity for glutamate uptake under conditions of oxidative stress, known to cause a significant decrease in glutamate clearance (Piani *et al.* 1993; Volterra *et al.* 1994b; Trotti *et al.* 1996; Agostinho *et al.* 1997; Sorg *et al.* 1997; Muller *et al.* 1998). Oxidative stress can be induced by ferrous ions (Schori *et al.* 2001). By culturing microglia in the presence of increasing concentrations of ammonium-ferrous (II) sulfate hexahydrate (0.01–0.1 mM), we showed that glutamate uptake was inhibited in a dose-dependent manner by the ferrous salt (Fig. 3). Activated T_{mbp} cells were nevertheless able to stimulate glutamate uptake even under these conditions of oxidative stress.

Further examination of the possible role of IFN-γ in the uptake of glutamate induced by activated T_{mbp} cells confirmed that this cytokine was indeed able to simulate, at least in part, the effect of activated T_{mbp} cells on microglia (Fig. 4a). The basal ability of microglia to take up glutamate was found to be 10% of the uptake ability of cultured astrocytes, whereas the ability of microglia treated with IFN-γ was as high as 40% of that in astrocytes (Fig. 4a). Primary cultured astrocytes are known to be potent scavengers of glutamate. Under our experimental conditions, addition of IFN-γ did not increase their potency (Fig. 4a).

Glutamate uptake is difficult to achieve in an environment of inflammation or oxidative stress (Trotti *et al.* 1996; Muller *et al.* 1998). We therefore examined the dual ability of microglia to scavenge extracellular glutamate and to produce nitric oxide. Because LPS is commonly used to activate microglia and to induce pro-inflammatory conditions (Lehnardt *et al.* 2003), we followed the uptake of glutamate

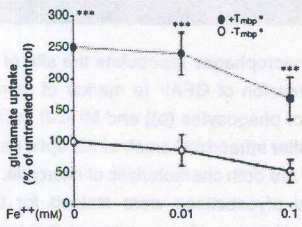
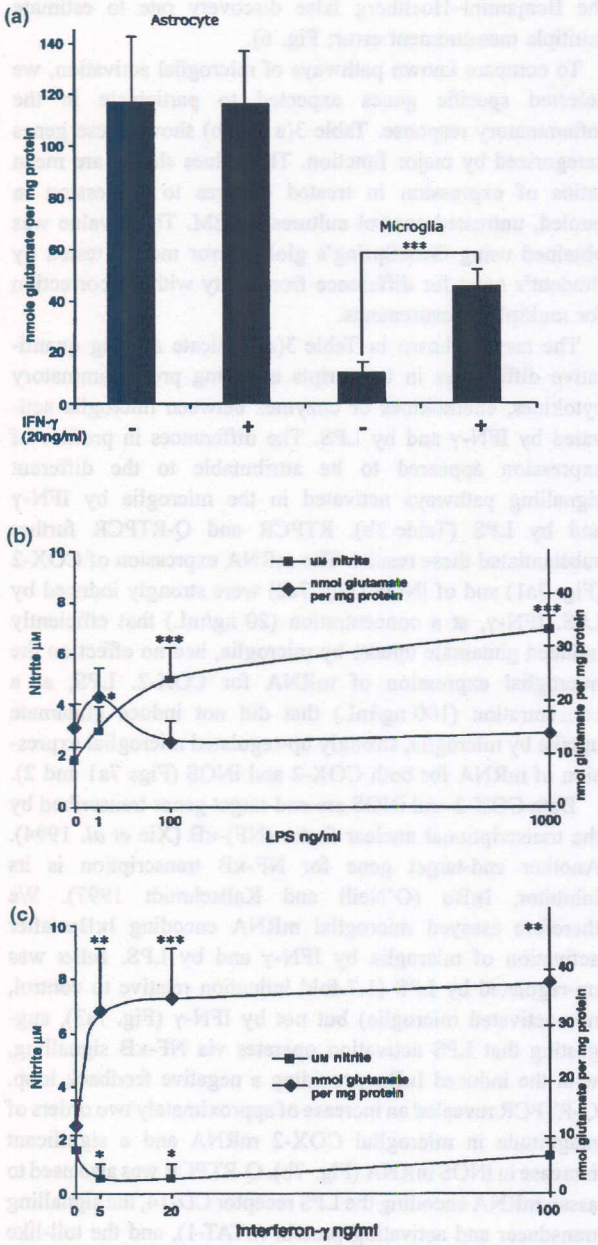


Fig. 3 Activated T_{mbp} cells enhance glutamate uptake of microglia-enriched cultures in an environment of oxidative stress. Cultures enriched in rat microglia were incubated for 24 h with the indicated concentration of ammonium-ferrous (II) sulfate hexahydrate (which increases the formation of toxic oxygen species), co-cultured with or without activated or non-activated T_{mbp} cells, and then incubated for 30 min at 37°C in uptake medium containing glutamate at a physiological concentration. Intracellular glutamate concentrations were determined as described in Materials and methods and are expressed as a percentage of the uptake in control cultures not treated with activated T_{mbp} cells (taken as 100%). Values are means ± SD of two separate experiments, each carried out with eight replicates.



and the production of NO by microglia activated by LPS. Treatment for 24 h with LPS (1–1000 ng/mL) (Fig. 4b) or IFN-γ (5–100 ng/mL) (Fig. 4c) had opposite effects on the ability of microglia to produce NO (Green *et al.* 1982) and to take up glutamate. While LPS at the above-mentioned concentration range did not alter glutamate uptake, it efficiently promoted NO production. IFN-γ, when added at 5 ng/mL, significantly induced the ability of microglia to remove extracellular glutamate, but did not up-regulate their NO production even at a concentration as high as 100 ng/mL.

Fig. 4 IFN-γ enhances the ability of microglia-enriched cultures but not of astrocytes to take up glutamate without promoting NO production. Rat microglia or astrocytes were incubated for 24 h with IFN-γ (20 ng/mL) and their intracellular uptake of glutamate was then determined. Glutamate uptake by astrocytes was almost 10-fold higher than that of microglia (expressed in nmol glutamate per mg protein). However, whereas IFN-γ had no effect on astrocyte uptake, it significantly affected the uptake of microglia (a). In a separate experiment, intracellular glutamate uptake and NO release were determined in cultures subjected for 24 h to treatments at the indicated concentration of LPS (b) or IFN-γ (c). Intracellular glutamate concentration was determined as described in Materials and methods and is presented as nmol glutamate/mg protein. Intracellular production of NO was assessed by the Griess method (Green *et al.* 1982) as the concentration of nitrite, a stable oxidative product of NO, in the culture supernatants and is expressed in µM. Values are means ± SD of three separate experiments, each carried out with six replicates.

Kinetics of glutamate uptake by microglia

Glutamate uptake by microglia with and without IFN-γ treatment was assayed as a function of time (between 10 and 60 min). At the indicated time points, microglia were washed well and their glutamate uptake was recorded (Fig. 5a). We also measured changes in the velocity of glutamate uptake (V_{max}) after treatment with IFN-γ (Fig. 5b).

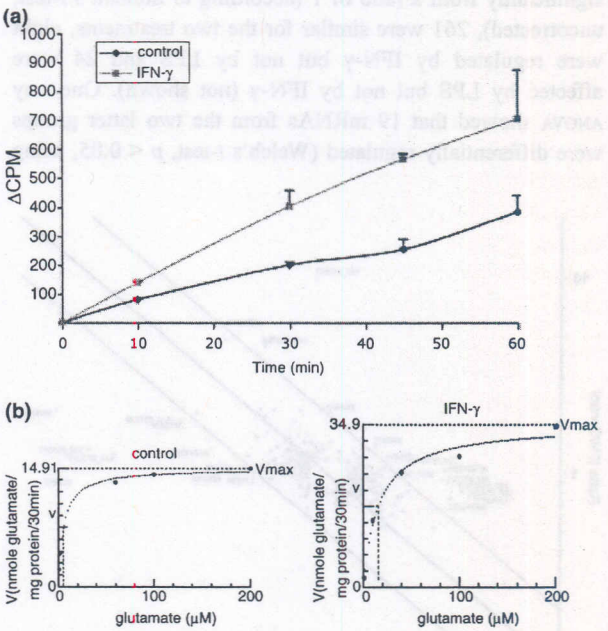


Fig. 5 Kinetics of glutamate uptake by microglia. Glutamate uptake by microglia, co-cultured with or without activated or non-activated T_{mbp} cells, was assayed as a function of time (between 10 and 60 min) (a) or during 30 min of incubation (b) at 37°C in uptake medium (see Materials and methods) containing [^{14}C]-glutamate at different concentrations (between 1 and 200 µM). Intracellular radioactivity was measured as described in Materials and methods. Data (means ± SEM) are recorded as Michaelis–Menten plots (b), which yield values of glutamate uptake velocity expressed as V_{max}.

Michaelis–Menten plots of the data, show that V_{max} (in nmol glutamate per mg protein per 30 min) of glutamate transport for IFN-γ-treated microglia was 34.9, compared with 14.9 for the corresponding microglia in the absence of IFN-γ.

Activation by IFN-γ and by lipopolysaccharide has different effects on microglial mRNA expression

IFN-γ and LPS may be regarded as representative, respectively, of adaptive and innate immune pro-inflammatory signalling (Bach *et al.* 1997; Boehm *et al.* 1997; Lehnardt *et al.* 2002, 2003). Therefore, the finding that glutamate uptake by microglia is increased by IFN-γ but not by LPS, and that IFN-γ can up-regulate microglial glutamate uptake without increasing NO production, prompted us to further compare the phenotypes of LPS- and IFN-γ-activated microglia. Using gene array analysis followed by RTPCR and Q-RTPCR, we assayed microglial transcripts encoding genes associated with the inflammatory response. To better compare mRNA regulation in microglia treated with LPS and with IFN-γ, we compared the treated microglia of each group with pooled, untreated microglial cultures on two-colour spotted oligonucleotide microarrays ($n = 3$ for each treated and control group). Of 297 mRNAs found to be expressed more than twice as frequently as in controls and differing significantly from a ratio of 1 (according to Student's *t*-test, uncorrected), 261 were similar for the two treatments, eight were regulated by IFN-γ but not by LPS and 24 were affected by LPS but not by IFN-γ (not shown). One-way ANOVA showed that 19 mRNAs from the two latter groups were differentially regulated (Welch's *t*-test, $p < 0.05$, using the Benjamini–Hochberg false discovery rate to estimate multiple measurement error; Fig. 6).

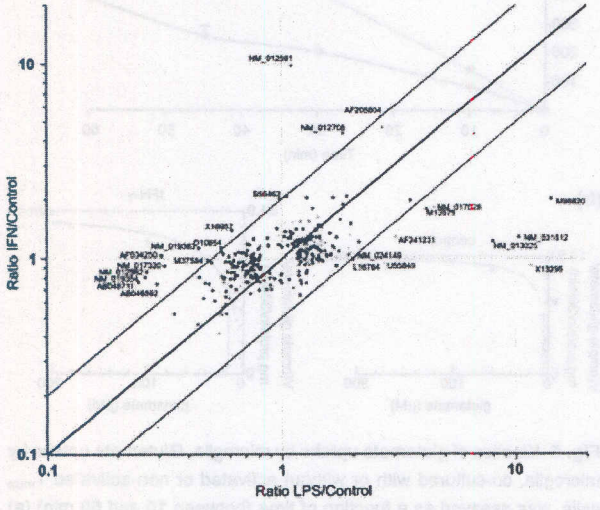


Fig. 6 Microarray analysis of the effects of LPS treatment and IFN-γ treatment of microglia-enriched cultures. For each treatment, the ratio of treated to untreated cultures is plotted. Labelled dots represent probes with ratios twice as large in one treatment as in the other (see text). The labels are GenBank accession numbers matching probe oligonucleotides.

To compare known pathways of microglial activation, we selected specific genes expected to participate in the inflammatory response. Table 3(a and b) shows these genes categorized by major function. The values shown are mean ratios of expression in treated cultures to expression in pooled, untreated control cultures ± SEM. The *p*-value was obtained using GeneSpring's global error model, tested by Student's *t*-test for difference from unity with no correction for multiple measurements.

The results shown in Table 3(a) indicate striking quantitative differences in transcripts encoding pro-inflammatory cytokines, chemokines or enzymes between microglia activated by IFN-γ and by LPS. The differences in profiles of expression appeared to be attributable to the different signalling pathways activated in the microglia by IFN-γ and by LPS (Table 3b). RTPCR and Q-RTPCR further substantiated these results. The mRNA expression of COX-2 (Fig. 7a1) and of iNOS (Fig. 7a2) were strongly induced by LPS. IFN-γ, at a concentration (20 ng/mL) that efficiently induced glutamate uptake by microglia, had no effect on the microglial expression of mRNA for COX-2. LPS, at a concentration (100 ng/mL) that did not induce glutamate uptake by microglia, strongly up-regulated microglial expression of mRNA for both COX-2 and iNOS (Figs 7a1 and 2).

Both COX-2 and iNOS are end-target genes transcribed by the transcriptional nuclear factor (NF)-κB (Xie *et al.* 1994). Another end-target gene for NF-κB transcription is its inhibitor, IκBα (O'Neill and Kaltschmidt 1997). We therefore assayed microglial mRNA encoding IκBα after activation of microglia by IFN-γ and by LPS. IκBα was up-regulated by LPS (1.7-fold induction relative to control, non-activated microglia) but not by IFN-γ (Fig. 7a3), suggesting that LPS activation operates via NF-κB signalling, with the induced IκBα providing a negative feedback loop. Q-RTPCR revealed an increase of approximately two orders of magnitude in microglial COX-2 mRNA and a significant increase in iNOS mRNA (Fig. 7b). Q-RTPCR was also used to assay mRNA encoding the LPS receptor CD-14, the signalling transducer and activating protein (STAT-1), and the toll-like receptor (TLR-4) in microglia. IFN-γ signalling appeared to focus on the JAK/STAT pathway, up-regulating STAT-1 by more than 3-fold relative to its up-regulation by LPS (IFN-γ, 3.8-fold induction relative to control; LPS, 1.1-fold induction relative to control). LPS activation appeared to propagate itself by up-regulating CD-14 (3.5-fold induction relative to control) but its quantitative effect on TLR-4 was only marginal. IFN-γ had no effect on TLR-4 expression.

Discussion

Microglia are the guardians and defenders of neuronal integrity in the CNS, yet they are also a source of

Table 3. Clusters of genes differently activated by LPS and IFN-γ.(a) pro-inflammatory, and (b) signaling genes. Results were extracted from data presented in Figure 4. Shown are probes selected by one-way ANOVA (Welch's t-test) at $p<0.05$ with Benjamini and Hochberg False Discovery Rate to estimate multiple measurement error. Selected results were hierarchically clustered using standard correlation. Each probe lists the corresponding GenBank accession number and short description.

(a)

Genbank	Gene description	IFN-γ	*P*-value	LPS	*P*-value
NM_01267	Tumor necrosis factor α	1.7 ± 0.4	NS	7.4 ± 0.6	0.000
NM_01701	Interleukin 1α	2.1 ± 0.9	NS	21.2 ± 1.2	0.000
M98820	Interleukin 1β	2.0 ± 0.6	NS	14.2 ± 0.8	0.000
NM_01258	Interleukin 6	1.5 ± 0.5	NS	13.8 ± 4.5	0.001
M46536	GRO	1.7 ± 0.1	NS	31.4 ± 3.1	0.000
NM_01302	MIP-1α	1.2 ± 0.5	NS	8.0 ± 0.3	0.000
M57441	MCP-1	1.2 ± 0.2	NS	26.1 ± 0.8	0.000
U45965	MIP-2 precursor	1.9 ± 0.7	NS	29.2 ± 2.4	0.000
AF208230	MIP-1β	2.3 ± 1.4	NS	19.5 ± 0.4	0.000
AF058786	JE/MCP-1	1.3 ± 0.1	0.027	18.0 ± 2.5	0.000
U17035	MOB-1/IP-10	9.0 ± 0.5	0.000	12.3 ± 1.1	0.000
NM_01261	iNOS (NOS2a)	0.8 ± 0.1	NS	13.7 ± 1.8	0.000
U24441	Matrix metalloproteinase-9	1.0 ± 0.1	NS	6.8 ± 0.6	0.000
M60616	Matrix-metalloproteinase-13	1.1 ± 0.2	NS	3.5 ± 0.4	0.001

(b)

Genbank	Gene description	IFN-γ	P-value	LPS	P-value
NM_012591	Interferon regulatory factor 1	9.8 ± 0.5	0.000	1.1 ± 0.1	NS
AF205604	STAT-1	5.4 ± 0.2	0.000	2.6 ± 0.1	0.000
AJ243123	SOCS-1	6.7 ± 0.3	0.000	1.3 ± 0.1	0.035
AF201901	IFN-γ receptor	0.6 ± 0.1	0.004	0.1 ± 0.0	0.008
M80367	guanylate binding protein 2	9.2 ± 0.3	0.000	6.7 ± 0.3	0.000
AF087943	CD-14	1.5 ± 0.1	0.006	3.8 ± 0.6	0.001
X63594	RL/IF-1 (1κB)	1.1 ± 0.1	NS	2.2 ± 0.2	0.002
AF269247	Protease activated receptor 3	1.0 ± 0.1	NS	8 ± 1.5	0.000

inflammation and toxicity. In this study we sought to gain a better understanding of the ambivalent character of microglial activity. On the basis of the present findings, we suggest that a well-controlled dialogue with the adaptive immune system (represented by T cells) will shape the microglial phenotype in the way best suited to cope with degenerative conditions.

We describe how stand-by microglia recruited to the site of a CNS injury are challenged by the toxic conditions there, caused, for example, by an excess of glutamate and a paucity of astrocytes (which normally perform the task of glutamate removal).

Our results showed that activated Th cells can significantly increase the ability of microglia, not only to perform their usual immune-related function of antigen presentation, but also to clear away extracellular glutamate. We further showed that the T-cell-induced activation of microglia can be mediated, at least in part, via IFN-γ signalling, and that this mode of activation is quite different from that displayed by the potent microglial activator LPS. Stimulation by IFN-γ significantly increased the velocity of glutamate uptake by microglia.

Threatening exogenous agents, such as bacteria or viruses, activate receptors of the innate immune system, such as CD14 and TLRs (Elward and Gasque 2003), leading to up-regulation of various types of killing machinery. The result is massive production of enzymes, cyokines and chemokines known to be involved in the immune response, which acts to eliminate threatening micro-organisms. Such a response is associated with production of NO and prostaglandins (Aloisi 2001). The adaptive immune system, represented by Th1 and Th2 cells, further regulates, augments or terminates this immune response (Aloisi *et al.* 2000). Glutamate neurotoxicity, however, presents a different challenge. A response that employs the tools of the innate immune system to fight off infective agents is not needed here, and might even turn neurotoxic by imposing oxidative stress, inhibiting glutamate uptake and causing glutamate release (Volterra *et al.* 1994a). Consistent with this notion are the results of the present study showing that activated myelin-specific T cells shape a microglial phenotype that favours not only antigen presentation but also glutamate clearance, and that this phenotype is quite different from the cytotoxic phenotype triggered by LPS. In line with this finding is a recently published report of up-regulation of glutamate uptake in synaptosomes obtained from the spinal cord of rodents with experimental autoimmune encephalomyelitis (an animal model of multiple sclerosis) at the peak

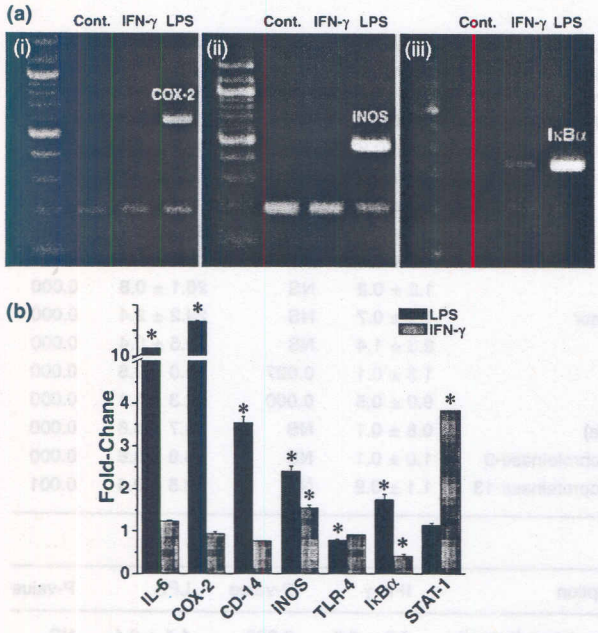


Fig. 7 LPS and IFN-γ activate different intracellular pathways. Rat microglia were incubated for 24 h with IFN-γ (20 ng/mL) or LPS (100 ng/mL). Specific mRNA was assayed by RTPCR (a) and Q-RTPCR (b), using selected primer pairs specific for the LPS receptor CD-14 and the toll-like receptor-4 (TLR-4), the oxidation-mediating enzyme inducible nitric oxide synthase (iNOS), the inflammation-mediating enzyme cyclooxygenase-2 (COX-2), the signalling transducer and activator protein STAT-1, and the inhibitor of the NF-κB transcription factor, IκBα. RTPCR products were assessed using 1.5% gel agarose (a). Q-RTPCR products were assessed by measuring the SYBR green fluorescence (see Material and methods). Values are means ± SD of three replicate determinations. Significance on Q-RTPCR, denoted by an asterisk, reflects difference from control levels detemined by ANOVA at $p < 0.05$, using the Benjamini–Hochberg false discovery rate to estimate multiple measurement error. (b)

of the disease, i.e. at the stage that correlates with the most abundant presence of activated autoimmune T cells at the lesion site (Ohgoh *et al.* 2002).

The effect of activated T_{mbp} cells on the uptake of glutamate by microglia was reproduced in this study by IFN-γ. Moreover, IFN-γ-activated microglia were capable of efficient glutamate uptake but did not produce NO, whereas LPS-activated microglia produced NO but did not take up glutamate. These two opposing phenotypes were further studied by examination of microglial mRNA expression after treatment of the microglia with IFN-γ and LPS using gene array analysis and PCR. The mRNA of cytokines, enzymes, and chemokines that are hallmarks of inflammation were strongly expressed in LPS-activated microglia, but not in microglia activated by IFN-γ. Among the cytokines induced in microglia by LPS are TNF-α, interleukin (IL)-1α, IL-1β and IL-6. The chemokines found in LPS-induced microglia are GRO, MCP-1, MIP-1α, MIP-2 and MIP-1β, and the enzymes are iNOS, COX-2, MMP-9 and MMP-13. We verified the gene array results by focusing on a few genes that might be relevant to glutamate imbalance and toxicity. The results confirmed that the enzyme iNOS, which contributes to oxidative imbalance, and the inflammation-mediating enzyme COX-2, were induced vigorously by LPS, but only weakly by IFN-γ. We also observed different modes of signalling by these two agents: LPS signalling was self-propagating and occurred via up-regulation of its receptor CD-14, whereas IFN-γ induced up-regulation of the signalling transducer and activator protein STAT-1, mediated via the well-characterized intracellular JAK-STAT signalling pathway. In addition, LPS signalling up-regulated IκBα, an inhibitor of the transcription factor NF-κB, whereas IFN-γ signalling did not.

Our results suggested that the clearance of glutamate by microglia was induced by high-affinity sodium-dependent transporters of the EAAT family. The specific transporter protein that is up-regulated has not yet been identified. Several alternative mechanisms for modulating EAAT activity have been suggested, including positive and negative feedback links to the mGlu receptor (Gegelashvili *et al.* 2001), activation of kinases such as PKA, PKC or PI3K (all implicated in up-regulating glutamate uptake; Davis *et al.* 1998), and the recently reported discovery of an EAAT-interacting protein that regulates glutamate uptake activity (Jackson *et al.* 2001; Lin *et al.* 2001). We found here that the amount and velocity of glutamate uptake by microglia were altered as a result of treatment with IFN-γ. Microglia have been shown to be a source of glutamate release and toxicity (Bal-Price and Brown 2001), acting at least in part via reverse transport (Noda *et al.* 2000). Metabolic failure and energy depletion could convert microglia from friendly glutamate scavengers to an unwelcome source of neurotoxicity. Our data suggest, however, that, whereas LPS is a likely candidate for triggering glutamate release, adaptively regulated microglia are likely to have the opposite effect. This notion was substantiated by preliminary findings (using a fluorometric glutamate detection assay), suggesting that LPS-treated microglia release significantly larger amounts of glutamate than untreated microglia, whereas treatment with IFN-γ does not affect glutamate release (data not shown). The participation of metabolic enzymes (Shaked *et al.* 2002), possibly up-regulated by IFN-γ cannot be ruled out.

Further studies are needed to fully elucidate the mechanism by which IFN-γ up-regulates glutamate uptake. IFN-γ is produced mainly by lymphocytes (Th1). The physiological relevance of the present finding is supported by observations by our group that systemic injection of activated T cells after injury to CNS axons results in co-localization of MHC-II-expressing activated microglia and T cells delineating the lesion site (Butovsky *et al.* 2001). Moreover, the fact that

T cells are locally activated was recently demonstrated by an assay of the state of activation of T cells isolated from the injured spinal cord, which confirmed that activated T cells could be recovered as early as 3 days after the injury (Lewitus *et al.*, unpublished). As adaptive immunity comes into play at a relatively late stage of an immune response, it is likely that IFN-γ signalling will be secondary to a primary (innate) stimulus.

In many studies, microglia are treated with IFN-γ in conjunction with LPS, which increases their toxicity. In our view, however, such studies only demonstrate how adaptive immunity tackles infection and not how the immune system fights off threats from within (as in neuronal degeneration). This contention is supported by the reported comparison of several cytokines with respect to their influence on glutamate uptake: glutamate uptake by astrocytes is inhibited in a dose-dependent manner by interleukin (IL)-1α or TNF-α. IFN-γ, when administered by itself, stimulates glutamate uptake, but inhibits it when administered in combination with IL-1α (Hu *et al.* 2000). In the present study, however, we were unable to detect any significant changes in the ability of astrocytes to take up glutamate after treatment with IFN-γ. The discrepancy might be because of species differences (human vs. rat) or glutamate uptake conditions. Nevertheless, our present demonstration that the ability of rat-cultured microglia to take up glutamate is of the same magnitude as that of astrocytes is in line with the reported finding that the ability of human macrophages to take up glutamate (K_m and V_{max}) is similar to that of primary cultured astrocytes (Rimaniol *et al.* 2000). Moreover, the induced ability of microglia to take up glutamate might be of biological significance: the capacity of microglia for glutamate uptake was found in this study to be up to 10% of that in cultured astrocytes, and as high as 40% in microglia treated with IFN-γ.

After injury to the CNS, microglia can 'sense' glutamate stress via their metabotropic glutamate receptors, G-protein-coupled receptors that signal information about extracellular glutamate status (Heuss *et al.* 1999; Scanziani 2002). Interestingly, metabotropic glutamate receptors are also expressed by T cells (Storto *et al.* 2000). There is an additional aspect to the ability of immune cells such as microglia and T cells to sense extracellular glutamate (possibly via metabotropic glutamate receptors) and respond to it (possibly by transporter-mediated uptake). Extracellular glutamate inhibits antigen presentation, and hence also T-cell activation, by inhibiting the cysteine exchanger and imposing oxidative stress (Angelini *et al.* 2002). On the basis of the present results, we suggest that the microglia-induced ability to eliminate glutamate also contributes indirectly to antigen presentation, which in turn successively promotes T-cell activation, IFN-γ secretion and further glutamate clearance. Our group recently proposed that antigen presentation is a necessary step in the protective immune response (Butovsky *et al.* 2001; Shaked *et al.* 2004), and that in the CNS parenchyma it is carried out mainly by microglia.

Glutamate is evidently not the only neurotransmitter whose metabolism and transport are regulated by cytokines. It was recently suggested that the transport of GABA, a major inhibitory amino acid, is facilitated by IL-4, a typical Th2 cytokine (Sholl-Franco *et al.* 2002). It is therefore likely that well-controlled adaptive immunity contributes to the shaping of microglial activity, not only via its Th1 arm, but also via additional arms such as Th2 and regulatory T cells (Hauben *et al.* 2000b; Kipnis *et al.* 2002). Preliminary results in our laboratory support the contention that the effect of IL-4 complements that of IFN-γ in the context of protective autoimmunity (Butovsky *et al.* unpublished observations).

Microglia constitute only 5–15% of the non-neuronal population of brain cells (Carson 2002), but as the cellular sentinels of the CNS they could be the most dominant population to respond and occupy the lesion site. This notion is consistent with the recently reported findings that the glutamate transporter GLT-1 is strongly expressed in activated microglia after nerve axotomy (Lopez-Redondo *et al.* 2000), and that microglia can play an important compensatory role in the early regulation of extracellular glutamate after a controlled cortical impact injury in the rat (van Landeghem *et al.* 2001). We suggest that the multiphenotypic character of microglia enables these cells to engage in a neuro-immune dialogue aimed at preserving neuronal homeostasis, yet their ability to display different and, at times apparently opposing, phenotypes demands close surveillance. Such regulation, which is provided by the adaptive immunity, is present under physiological conditions, and is absent or deficient in pathological situations. It supplies the underlying rationale of T-cell-based therapeutic vaccination for neurodegenerative conditions, a therapy that helps the body to cure itself (Hauben and Schwartz 2003).

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