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# IFN-γ and IL-4 differentially shape metabolic responses and neuroprotective phenotype of astrocytes

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**Abstract**

Astrocytes can either exacerbate or ameliorate secondary degeneration at sites of injury in the CNS but the contextual basis for eliciting these opposing phenotypes is poorly understood. In this study, we demonstrate that the two major cytokines produced by Th1 and Th2 cells, interferon-γ (IFN-γ), and interleukin-4 (IL-4), respectively, contribute differentially to shaping a neuroprotective response in astrocytes. While IFN-γ protects the ability of oxidatively stressed murine astrocytes to clear extracellular glutamate in culture, IL-4 has no effect at any concentration that was tested (10–100 ng/mL). The enhanced release of neuroprotective thiols and lactate by astrocytes in response to T cell stimulation is mimicked by both IL-4 and IFN-γ. When co-administered, IL-4 abrogated the protective effect of low IFN-γ on the glutamate clearance function of oxidatively stressed astrocytes in a dose-dependent manner. Astrocyte-conditioned media obtained from cells cultured in the presence of IL-4 (10 or 100 ng/mL) or IFN-γ (10 ng/mL) decreased by ~2-fold, neuronal apoptosis induced by oxidative stress *in vitro*. However, unlike IL-4, IFN-γ at high concentrations (100 ng/mL) was not neuroprotective. Our studies with IFN-γ and IL-4 suggest that a balanced Th1 and Th2 cytokine response might be needed for protecting two key astrocytic functions, glutamate clearance and thiol secretion and might be pertinent to neuroprotective approaches that are aimed at inhibition of an initial pro-inflammatory response to injury or its sustained boosting.

**Keywords:** astrocytes, neuroprotection, glutamate, interferon-γ, interleukin-4, redox.

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Historically, T cell-mediated inflammation in the CNS has been viewed as being detrimental to the outcome of neural injury. However, this view has been challenged by reports that T cells play a critical role in neuroprotection after CNS injury (Moalem *et al.* 1999b; Hauben *et al.* 2000a; Kipnis *et al.* 2002a). The concept of ‘protective autoimmunity’ originated from the observation that autoimmune T cells directed against CNS antigens increased neuroprotection in an optic nerve crush injury model (Moalem *et al.* 1999a) and spinal cord contusion (Hauben *et al.* 2000b). The beneficial role of T cells in CNS injury was further supported by the observation that T cell-deficient mice exhibited a poorer outcome for CNS injury than wild type controls and that replenishing T cells increased neuronal survival (Kipnis *et al.* 2001; Yoles *et al.* 2001). Follow-up studies have sought to exploit the therapeutic potential of this concept for a variety of neurodegenerative disorders including spinal cord injury (Hauben *et al.* 2001), Alzheimer’s disease (Janus *et al.* 2000), amylotrophic lateral sclerosis (Angelov *et al.* 2003), and Parkinson’s disease (Benner *et al.* 2004).

A further complication in understanding the role of T cells in the context of CNS injury is that opposing effects of these cells on injury outcome have been reported. Thus, studies aimed at elucidating the differential effects of Th1 and Th2 subtypes on neuronal survival have furnished contrasting results (Gimsa *et al.* 2001; Kipnis *et al.* 2002a; Wolf *et al.* 2002). In a rodent entorhinal–hippocampal brain slice culture model, Th2 cells were reported to be significantly more neuroprotective than Th1 cells (Gimsa *et al.* 2001; Wolf *et al.* 2002). On the other hand, switching the microglial

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*Abbreviations used*: Cys-Gly, cysteinyl-glycine; HBSS, Hanks’ balanced salt solution; IFN-γ, interferon-γ; IL-4, interleukin-4; ROS, reactive oxygen species; *t*-BuOOH, tertiary butyl hydroperoxide; TUNEL, terminal deoxynucleotidyl transferase-dUTP nick end labeling.

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phenotype from neurotoxic to neuroprotective upon interaction with T cells was reported to involve secretion of interferon-γ (IFN-γ), a Th1 cytokine (Shaked *et al.* 2005). We have recently demonstrated that $CD3^+$ T cells, by secreting glutamate and enhancing the glutamate clearance capacity of astrocytes, induce the latter to release the neuroprotectants, lactate and cysteine, important for neuronal defense against oxidative injury (Garg *et al.* 2008). Glutamate clearance by astrocytes was enhanced by IFN-γ and interleukin-2 (IL-2) alone but not by IL-4 (Garg *et al.* 2008) laying the groundwork for this study in which the effect of different concentrations of these two cytokines on thiol and lactate release and on protecting the glutamate uptake function of astrocytes have been assessed.

Astrocytes support neuronal function in many ways, e.g. by providing guidance cues and neurotrophic factors (Oren *et al.* 2004), helping to maintain osmolarity, ionic composition and pH, and affecting the strength and number of synapses (Rosenberg *et al.* 2001; Abbott 2002). Astrocytes tightly ensheath neuronal synapses and play a critical role in the glutamate–glutamine cycle that is integral to glutamatergic neurotransmission (Aschner *et al.* 2001; Xu *et al.* 2001; Kintner *et al.* 2004). By supplying neurons with lactate (Pellerin and Magistretti 1994; Bouzier-Sore *et al.* 2002; Gladden 2004) and cysteine (Dringen 2000), astrocytes contribute to neuronal energy metabolism (Sokoloff *et al.* 1996) and to antioxidant capacity. Impaired astrocytic function has major consequences on neuronal function. For example, under pathological conditions, enhanced production of reactive oxygen species (ROS) compromises the intrinsic antioxidant capacity of astrocytes and neurons and leads to oxidative stress and cell demise (Tacconi 1998). Several neurodegenerative disorders are characterized by major perturbations in the glutathione system (Calabrese *et al.* 2005; Sarandol *et al.* 2007). Accumulation of glutamate in the CSF is one of the major contributors of secondary degeneration following CNS injury and results in part from compromised glutamate clearing capacity of astrocytes when ROS levels increase (Brand *et al.* 1999; Tilleux and Hermans 2007).

In this study, we have tested the hypothesis that an initial proinflammatory response to CNS injury is required to increase neuronal survival under oxidative stress conditions using cytokines as surrogates of the T cell subtypes. Our studies support the model that an initial response to low IFN-γ, a Th1 cytokine, protects astrocytic glutamate clearance capacity that is compromised under oxidative stress conditions and induces release of neuroprotectants (thiols and lactate). However, these effects are either inhibited or are marginally protected at high concentrations of IFN-γ. In contrast, the Th2 cytokine, IL-4, induces release of the same neuroprotectants in a dose-dependent fashion, but does not protect the glutamate clearing function of oxidatively stressed astrocytes. Co-administration of the two cytokines results in a dose-dependent abrogation by IL-4 of the protective effect of low IFN-γ on glutamate clearance by oxidatively stressed astrocytes, suggesting that a balance between these cytokines might be important for shaping a neuroprotective response.

## Materials and methods

### Isolation and preparation of murine primary cells

The protocols used for handling animals were approved by the University Committee on Use and Care of Animals, University of Michigan.

#### *Astrocytes*

Primary murine cortical astrocytes cultures were prepared as described previously (Garg *et al.* 2008). At the end of the third passage, cells were seeded in a 24-well plate at a density of $5 \times 10^5$ cells/well in 1 mL media and incubated for a week (with half the medium being changed every third day) prior to being used for experiments. To check the purity of astrocytes and possible neuronal and microglial contamination in our cell preparation, cortical cells were labeled after the third passage with anti-CD11b (1 : 1000 dilution), anti-beta III tubulin (TUJ1, 1 : 1000 dilution), or anti-glial fibrillary acidic protein (1 : 1000 dilution) monoclonal antibodies (Chemicon-Millipore, Temecula, CA, USA), which are markers for microglia, neurons, and astocytes, respectively. The population of antibody-positive cells under confocal fluorescence microscopy (for all three cells types) and fluorescence-activated cell sorting (for astrocytes and microglia) was determined. While microglial contamination was not detected, neuronal contamination at ≤7% was observed. Based on this analysis, the purity of astrocytes was estimated to be ≥93% (data not shown).

#### *T lymphocytes*

Lymph nodes (axillary, inguinal, superficial cervical, mandibular, and mesenteric) and spleens were harvested and mashed and $CD3^+$ T cells were purified as described previously (Kipnis *et al.* 2004). Purified $CD3^+$ T cells were cultured in 24-well plates at a density of $1 \times 10^6$ cells/mL supplemented with 0.5 μg/mL anti mouse-CD3 antibody (R&D Systems, Minneapolis, MN, USA) in the presence of 3000 rad-irradiated syngeneic splenocytes, serving as antigen presenting cells. After 3 days of incubation, cells were collected, washed by low speed centrifugation (150 *g*) to remove dead splenocytes, and the T cells were resuspended in fresh medium for co-culture experiments with astrocytes.

#### *Neurons*

Dissociated neurons were prepared from embryonic days 13 to 15 mice and cultured in Neurobasal media containing 1x B27 supplement, 1x N2 supplement (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, and penicillin–streptomycin (10 000 U/mL) on laminin (Invitrogen) and poly-D-lysine (Sigma, St Louis, MO, USA) coated glass coverslips (12 mm; Bellco Glass, Vineland, NJ, USA) in 24-well plates as described previously (Garg *et al.* 2008). Cells were cultured at a density of 250 000 cells/well for 11 days in a humidified incubator with 5% $CO_2$ at 37°C prior to terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) staining.

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### Culture conditions

Astrocytes were replenished with fresh media and incubated with or without different concentrations of IL-4 or IFN-γ (mouse; R&D Systems) as specified in the Figure Legends. The half maximal effective dose ($ED_{50}$) reported by the manufacturer for mouse rIL-4 was 1–2 ng/mL and for mouse rIFN-γ was 0.3–0.9 ng/mL [a cell proliferation assay using a mouse IL-4 responsive cell line, HT-2, was used to report the biological activity of recombinant mouse IL-4 (Kappler *et al*. 1981). An antiviral assay using L-929 cells infected with EMC virus was used to report the activity of recombinant mouse IFN-γ (Vogel *et al*. 1982)]. At the indicated time points, aliquots of the culture media were collected and used for analysis of extracellular thiols [cystine, cysteine, and cysteinyl-glycine (Cys-Gly)] and lactate. To analyze the role of different cytokines on glutamate clearance by astrocytes under normoxic and oxidatively stressed conditions, cells were incubated with either 500 μM glutamate or 500 μM glutamate + 800 μM tertiary butyl hydroperoxide (*t*-BuOOH) and with or without IL-4 or IFN-γ. At the indicated time points, aliquots of the culture media were collected and the concentration of glutamate remaining in the medium was determined by HPLC as described previously (Garg *et al.* 2008). Anti-IFN-γ antibody when used was administered as a bolus at a final concentration of 10 μg/mL. For co-culture experiments, astrocytes and $CD3^+$ T cells were incubated at a 1 : 1 ratio and aliquots of culture media were used for thiols and glutamate analysis by HPLC. To check the effect of mixed cytokines on astrocytic glutamate clearance under oxidative stress conditions, cells were incubated with 500 μM glutamate + 800 μM *t*-BuOOH with or without IL-4 or IFN-γ alone (10 ng/mL or 100 ng/mL each) or a combination of both cytokines at different concentration as indicated in figure legend. At the indicated time points, aliquots of the culture media were collected and the concentration of glutamate remaining in the medium was determined by HPLC as described above.

For analysis of intracellular glutathione, cells were washed three times with ice-cold phosphate-buffered saline, resuspended in phosphate-buffered saline, and then detached by gentle scraping on ice. An aliquot of the cell suspension was mixed with an equal volume of metaphosphoric acid solution (16.8 mg/mL $HPO_3$, 2 mg/mL EDTA, and 9 mg/mL NaCl) and centrifuged at 12 000 *g* for 10 min at 4°C. Cell lysate supernatants were saved and stored at −80°C until further use. To measure protein concentration, an aliquot of the cell suspension was mixed with an equal volume of lysis buffer (0.1 M sodium phosphate, pH 7.4, containing 0.1% Triton X-100, 10 μL/mL protease inhibitor cocktail (Sigma), 25 μg/mL tosyllysine chloromethylketone, and 5 μg/mL phenylmethylsulfonyl fluoride (Sigma) and centrifuged at 12 000 *g* for 10 min at 4°C. The protein concentration in the cell lysate was measured by the Bradford method (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard.

### Metabolite analysis

Extracellular cysteine, cystine, total Cys-Gly, total glutathione, and glutamate concentrations were measured as described previously (Garg *et al.* 2008). The concentration of metabolites in the medium and in $CD3^+$ T cell-conditioned medium was subtracted from the final values. The concentration of each metabolite was determined using a calibration curve generated for that compound.

### Glutamate analysis

Extracellular glutamate concentration was also measured by using an enzymatic method (glutamate–glutamine kit; Sigma) as per the manufacturer's protocol as described previously (Garg *et al.* 2008).

### Lactate assay

Extracellular lactate concentration in the culture supernatants was measured using the Lactate Assay Kit (BioVision, Mountain View, CA, USA) as per the manufacturer's protocol. Optical density was measured in a 96-well plate reader at 570 nm. Lactate concentrations in the samples were determined using a calibration curve generated for standards of known concentration. The background value for media (blank) was subtracted for the final measurements.

### Reactive oxygen species determination

Intracellular ROS in live astrocytes were detected using the Image-iT Live Green Reactive Oxygen Species Detection Kit as per the manufacturer's protocol (Molecular Probes, Eugene, OR, USA). In brief, astrocytes were incubated either with $CD3^+$ T cells (at a 1 : 1 ratio) or with IFN-γ (20 ng/mL) for 2 h at 37°C. Cells were washed gently with Hanks' balanced salt solution (HBSS) pre-warmed at 37°C followed by labeling with 25 μM carboxy-$H_2$DCFDA [5-(and-6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate; Invitrogen] for 1 h at 37°C. Cells were washed twice with HBSS, trypsinized to detach from the plate, and collected in tubes followed by two more washes with HBSS. Finally, cells were suspended in HBSS containing 0.2% bovine serum albumin on ice. Fluorescent excitation/emission maxima at 495/529 were recorded using a fluorescence-activated cell sorting caliber instrument.

### Terminal deoxynucleotidyl transferase-dUTP nick end labeling assay

$CD3^+$ T cell-primed astrocyte-conditioned media was shown to protect neurons against oxidative stress death (Garg *et al.* 2008). To test the effect of Th1 versus Th2 cytokine-primed astrocytes on neuroprotection, neuronal apoptosis was measured by incubating primary neurons with either media conditioned by astrocytes or astrocytes incubated with 10 ng/mL or 100 ng/mL of IL-4 or IFN-γ for 10 h at 37°C. Following incubation, 500 μL of each conditioned media was transferred onto a neuronal monolayer suspended in 250 μL of neuronal media in the presence or absence of 200 μM *t*-BuOOH for 10–12 h. To test the neuronal response to cytokines in the presence or absence of *t*-BuOOH, primary neurons were incubated with or without 10 ng/mL or 100 ng/mL of IL-4 or of IFN-γ for 10 h at 37°C in the presence or absence of 200 μM *t*-BuOOH. Apoptosis of the neurons was determined using the TUNEL staining (*In situ* cell death detection kit; Roche Diagnostics, Indianapolis, IN, USA) as per the manufacturer's protocol. For this, coverslips were mounted with mounting gel (Sigma). Nuclei were stained with Hoechst stain (excitation 365 nm and emission 480 nm) and quantitative analysis was performed by counting > 2000 cells and presented as percent apoptosis by dividing the total number of apoptotic cells (TUNEL positive) by the total number of cells (Hoechst positive) in that frame.

### Statistics

Comparison between groups was performed using Student's *t*-test. A value of $p < 0.05$ was considered to be statistically significant.

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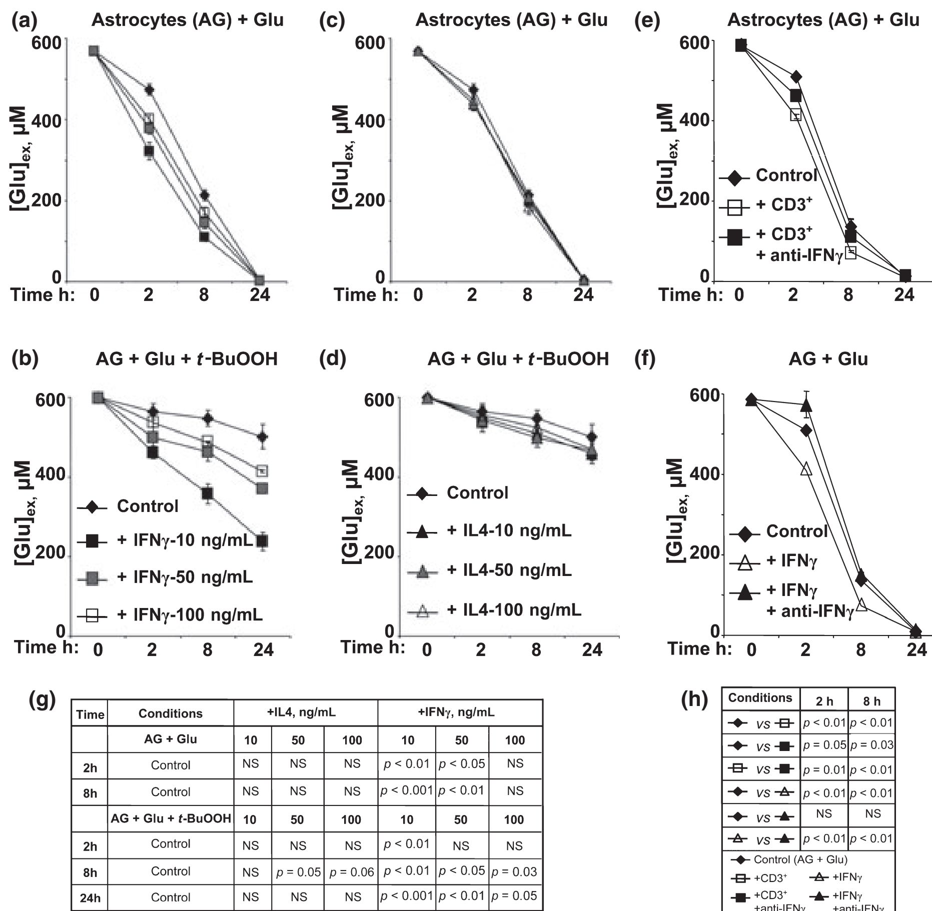
## Results

### IFN-γ enhances the glutamate clearance capacity of astrocytes under normoxic and toxic conditions

We have previously shown that activated $CD3^+$ T cells enhance the glutamate clearance capacity of astrocytes and that this response is stimulated by IFN-γ and IL-2 but not by IL-4 when applied at a concentration of 20 ng/mL each (Garg *et al.* 2008). We have determined the dose dependence of the IFN-γ effect and examined whether either higher or lower concentrations of IL-4 than previously used, can influence glutamate clearance by astrocytes. To test this, astrocytes were cultured with 500 μM glutamate added to the medium in the presence or absence of IL-4 or IFN-γ (at concentrations of 10, 50, and 100 ng/mL each). Under normoxic conditions, high concentrations of IFN-γ (50 and 100 ng/mL) had a modest effect, enhancing by ~19% and ~14%, respectively, the basal glutamate clearance rate of astrocytes (51 μM/h) compared with an ~32% increase observed with 10 ng/mL IFN-γ (Fig. 1a). Under oxidative




| Time | Conditions | +IL4, ng/mL | | | +IFNγ, ng/mL | | |
|---|---|---|---|---|---|---|---|
| | AG + Glu | 10 | 50 | 100 | 10 | 50 | 100 |
| 2h | Control | NS | NS | NS | $p < 0.01$ | $p < 0.05$ | NS |
| 8h | Control | NS | NS | NS | $p < 0.001$ | $p < 0.01$ | NS |
| | AG + Glu + *t*-BuOOH | 10 | 50 | 100 | 10 | 50 | 100 |
| 2h | Control | NS | NS | NS | $p < 0.01$ | NS | NS |
| 8h | Control | NS | $p = 0.05$ | $p = 0.06$ | $p < 0.01$ | $p < 0.05$ | $p = 0.03$ |
| 24h | Control | NS | NS | NS | $p < 0.001$ | $p < 0.01$ | $p = 0.05$ |

| Conditions | 2 h | 8 h |
|---|---|---|
| ◆ *vs* □ | $p < 0.01$ | $p < 0.01$ |
| ◆ *vs* ■ | $p = 0.05$ | $p = 0.03$ |
| □ *vs* ■ | $p = 0.01$ | $p < 0.01$ |
| ◆ *vs* △ | $p < 0.01$ | $p < 0.01$ |
| ◆ *vs* ▲ | NS | NS |
| △ *vs* ▲ | $p < 0.01$ | $p < 0.01$ |

◆ Control (AG + Glu); □ $+CD3^+$; ■ $+CD3^+$ +anti-IFNγ; △ +IFNγ; ▲ +IFNγ +anti-IFNγ

**Fig. 1** The effect of cytokines on the glutamate clearing capacity of astrocytes. Comparison of the glutamate-clearing ability of untreated (a and c) and *t*-BuOOH-treated (b and d) astrocytes (AG) in the presence or absence of different concentrations of cytokines: astrocytes were incubated with 500 μM glutamate added to the cell culture medium (containing ~100 μM glutamate) ± 10, 50, 100 ng/mL each of IFN-γ (squares, a) or IL-4 (triangles, c) or 500 μM glutamate + 800 μM *t*-BuOOH ± 10, 50, 100 ng/mL each of IFN-γ (squares, b), or IL-4 (triangles, d). Panels (e and f) show that $CD3^+$ T cell-derived IFN-γ enhances glutamate clearance by astrocytes as neutralization with anti-IFN-γ antibody diminishes this effect. Astrocytes were incubated with 500 μM glutamate ± $CD3^+$ T cells (squares, e) or 10 ng/mL IFN-γ (triangles, f) and in the absence (open) or presence (filled) of anti-IFN-γ antibody (10 μg/mL). At the indicated time points, culture medium was collected and the concentration of extracellular glutamate was measured as described under Materials and methods. Results show the mean ± SD and are representative of three (a, b, c, and d) or two (e and f) independent experiments that were each performed in triplicate on different batches of cells. Statistical analysis using the Student's *t*-test revealed significant changes in glutamate clearance by astrocytes as a function of time and stimulation by IFN-γ but not by IL-4. Panels (g and h) show the statistical analyses of the difference in glutamate clearance as a function of cytokine stimulation versus unstimulated control (for data in panels a–d) and of the differences in glutamate clearance as a function of cytokine stimulation versus neutralizing antibody versus control (for data in panel e–f); ns = not significant.

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stress conditions induced by 800 μM *t*-BuOOH, IFN-γ protected astrocytes from almost complete abrogation of glutamate clearance capacity in a dose-dependent manner. Thus, glutamate clearance rates of ~15, ~8, and ~5 μM/h were observed with 10, 50, and 100 ng/mL IFN-γ, respectively (Fig. 1b). In contrast, IL-4 stimulation did not protect the glutamate clearance of astrocytes in comparison to control under either normoxic or toxic conditions (Fig. 1c and d). The stimulatory effect of $CD3^+$ T cells or IFN-γ on glutamate clearance by astrocytes was further confirmed by incubating astrocytes with 500 μM glutamate in the presence or absence of either $CD3^+$ T cells or IFN-γ and with or without anti-IFN-γ antibody. At different time points, aliquots of the culture medium were collected and residual glutamate in the medium was measured. The stimulatory effect of $CD3^+$ T cells or IFN-γ on glutamate clearance by astrocytes was inhibited by the presence of an IFN-γ neutralizing antibody, confirming the role of this cytokine in the process (Fig. 1e and f). The inhibition was partial, indicating that other factors derived from $CD3^+$ T cells also contribute to modulating this astrocytic function. The statistical analyses for the data in Fig. 1 a–d and e–f are shown in Fig. 1g and h, respectively.

### IL-4 antagonizes the IFN-γ-mediated glutamate clearance function of oxidatively challenged astrocytes

To assess whether the presence of IL-4 can negate the protective effect of IFN-γ on glutamate clearance by oxidatively stressed astrocytes, astrocytes were incubated with *t*-BuOOH and glutamate and cytokine mixtures present at different ratios. At the indicated time points, the glutamate remaining in the culture medium was measured. The enhanced glutamate clearance observed with10 ng/mL IFN-γ stimulation of astrocytes was inhibited by the presence of IL-4 in a dose-dependent manner (Fig. 2). When IL-4 and IFN-γ were present at high concentrations, the protective effect on glutamate clearance was lost and the extracellular glutamate level was comparable to that in the control (Fig. 2).

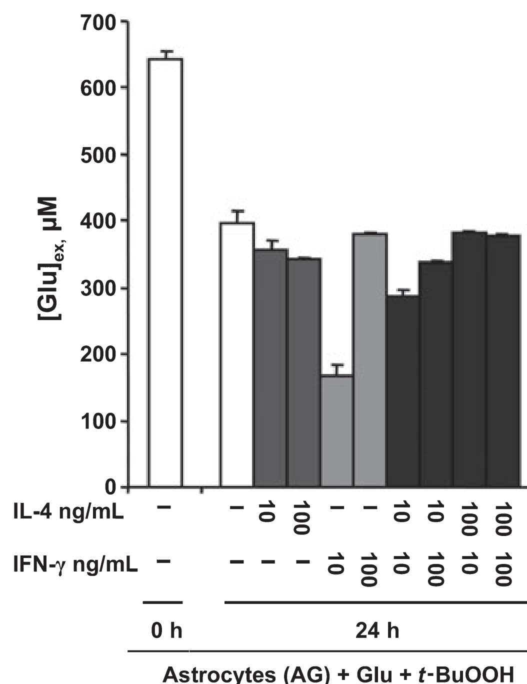


**Fig. 2** IL-4 antagonizes the IFN-γ-mediated protection of glutamate clearance by oxidatively challenged astrocytes. Astrocytes were incubated with 500 μM glutamate + 800 μM *t*-BuOOH with no other additions (white bar), with IL-4 (dark gray bars) with IFN-γ (light gray bars) alone (10 and 100 ng/mL each) or with a mixture of both cytokines (black bars) at different concentrations. At the indicated time points, aliquots of the culture media were collected and the concentration of glutamate remaining in the medium was determined by HPLC as described under Materials and methods. The results show the mean ± SD and are representative of two independent experiments that were each performed in duplicate on different batches of cells. Statistical analysis using the Student's *t*-test revealed significant changes in glutamate clearance by astrocytes as a function of time and stimulation by IFN-γ but not by IL-4 and that the presence of IL-4 abrogates the IFN-γ-enhanced glutamate clearance by astrocytes.

### IFN-γ and IL-4 mediate cysteine release from astrocytes in a dose-dependent manner

Under normoxic conditions, astrocytes are known to secrete neuroprotective metabolites such as cysteine, which is needed for the biosynthesis of glutathione in neurons (Dringen 2000). We have assessed the effect of IFN-γ versus IL-4 on the magnitude of cysteine release (Fig. 3a). Both IL-4 and IFN-γ (at a final concentration of 10 ng/mL each) enhanced cysteine release from astrocytes in a time-dependent manner compared with untreated controls. Extracellular cysteine levels in untreated astrocytes increased from 4.1 ± 0.1 μM to 10.5 ± 0.5 to 11.8 ± 0.1 μM at 2, 8, and 24 h, respectively. Stimulation with IL-4 (10 ng/mL) increased extracellular cysteine to 4.7 ± 0.06 μM (2 h), 13.0 ± 0.2 μM (8 h), and 15.2 ± 0.03 μM (24 h). Stimulation with IFN-γ (10 ng/mL) increased extracellular cysteine to 5.8 ± 0.7 μM (2 h), 19.1 ± 0.9 μM (8 h), and 20.2 ± 0.4 μM (24 h). Next, we examined the effect of higher doses (50 and 100 ng/mL) of IL-4 and IFN-γ on astrocytic cysteine release. IFN-γ was very inhibitory at concentrations of 50 and 100 ng/mL unlike its effect at 10 ng/mL. In fact, when astrocytes were treated with high concentrations of IFN-γ, the extracellular cysteine level was lower than in untreated controls. It is not known whether the decrease in extracellular cysteine resulted from its increased utilization or its enhanced oxidation. In contrast, IL-4 was found to stimulate cysteine accumulation at all three concentrations. The role of $CD3^+$ T cell-derived IFN-γ on cysteine release was confirmed by neutralizing the cytokine using anti-IFN-γ antibody, which significantly inhibited $CD3^+$ T cell-induced cysteine accumulation (Fig 3b).

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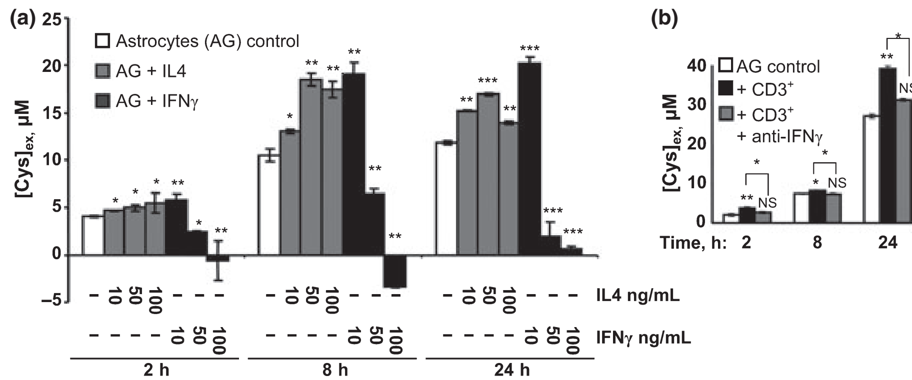


**Fig. 3** Dose-dependent effect of cytokines on astroglial cysteine release. (a) Culture medium from murine astrocytes incubated alone (open bar), or with 10, 50, and 100 ng/mL each of IL-4 (gray bar) or IFN-γ (black bar) was collected at the indicated time points and the concentration of cysteine was analyzed. The figure is representative of three independent experiments and shows mean ± SD. Panel (b) demonstrates that IFN-γ derived from CD3+ T cells mediates cysteine release from astrocytes as neutralization with anti-IFN-γ antibody inhibits cysteine accumulation. Astrocytes were incubated with or without CD3+ T cells in the absence (black bar) or in the presence (gray bar) of anti-IFN-γ antibody (10 μg/mL) as described under Materials and methods. Results show the mean ± SD of three independent experiments (a) or mean ± SD representative of two independent experiments (b) that were each performed in triplicate (a) or in duplicate (b) on different batches of cells. Statistical analysis of the difference in cysteine release as a function of cytokine stimulation (filled bar) versus control (open bar) is shown; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant.

## IFN-γ and IL-4 mediate Cys-Gly and glutathione release from astrocytes

As neurons transport cystine inefficiently, glutathione synthesis in these cells appears to be dependent on the provision of cysteine by astrocytes (Dringen 2000). The route by which cysteine is made available to neurons is by secretion of glutathione by astrocytes and its subsequent cleavage by γ-glutamyl transpeptidase to Cys-Gly and by a dipeptidase, to cysteine. We have therefore assessed the effects of IL-4 and IFN-γ on the extracellular cystine, Cys-Gly and glutathione pools (Fig. 4) Compared with the values for untreated controls ([Cys-Gly] of 3.7 ± 0.3 and 16.7 ± 0.1 μM at 2 and 24 h and [glutathione] of 0.8 ± 0.1 and 12.8 ± 1.6 μM at 2 and 24 h), IL-4 at a concentration of 10 ng/mL, enhanced Cys-Gly and glutathione concentrations in a time-dependent manner. Thus, Cys-Gly concentration increased by ~116% and ~40% and glutathione by ~76% and ~50% (at 2 and 24 h, respectively). At a concentration of 100 ng/mL, IL-4 increased Cys-Gly levels by ~150% and ~60% and glutathione levels to ~97% and ~70% (at 2 and 24 h, respectively) in comparison to untreated controls (Fig. 4a and b). Although IFN-γ also enhanced the extracellular concentrations of both thiols, it was more effective at a lower concentration. Thus, Cys-Gly levels increased by ~110% and ~60% while glutathione levels increased by ~86% and ~65% (at 2 and 24 h, respectively) with 10 ng/mL IFN-γ; and Cys-Gly levels increased by ~50% and ~20% and glutathione levels increased by ~45% and ~26% at the same time points with 100 ng/mL IFN-γ compared with untreated controls (Fig. 4a and b).

In principle, the observed increase in cysteine, or Cys-Gly could result from stimulation of extracellular cystine uptake

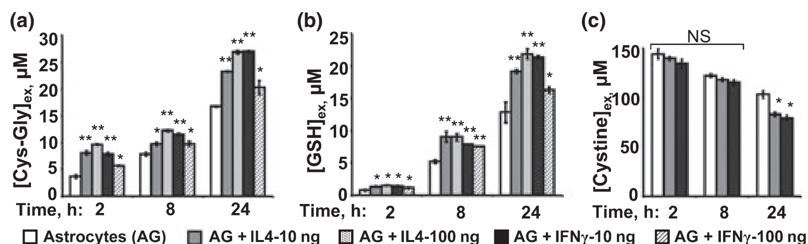


**Fig. 4** Dose-dependent effect of IL-4 or IFN-γ on astroglial Cys-Gly and glutathione (GSH) release. Murine astrocytes incubated alone (open bar), with 10 ng/mL IL-4 (gray bar) or 100 ng/mL IL-4 (dotted gray bar), with 10 ng/mL IFN-γ (black bar), or 100 ng/mL IFN-γ (striped black bar). At the indicated time points, the culture media were collected and the concentration of Cys-Gly (a), GSH (b), and cystine (c) were determined as described under Materials and methods. The results represent the mean ± SD of two independent experiments that were each performed in triplicates on different batches of cells. Statistical analysis using the Student's *t*-test revealed significant changes in Cys-Gly and glutathione secretion and cystine consumption by astrocytes as a function of time and treatment with either cytokines versus the untreated control; *$p < 0.01$; **$p < 0.001$; ns, not significant.

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and/or activation of the transsulfuration flux, which provides an intracellular route for cysteine biosynthesis from methionine. We found that both cytokines enhanced utilization of extracellular cystine by astrocytes between 2 and 24 h (144 ± 5 μM → 103 ± 3 μM in untreated, 139 ± 2 μM → 83± 2 μM in IL-4 treated, and 134 ± 5 μM → 79 ± 3 μM in IFN-γ treated astrocytes) (Fig. 4c). As the degree of cysteine and glutathione accumulation by astrocytes is qualitatively correlated with cystine consumption from the media, it suggests that extracellular cysteine may be derived from extracellular cystine. However, these results do not rule out the potential contribution of the transsulfuration pathway.

### IFN-γ and IL-4 increase intracellular glutathione concentration in astrocytes

As the extent of glutathione secretion is dependent on the intracellular glutathione content (Dringen 2000), we determined the effect of cytokines on the astrocytic glutathione pool size. The intracellular glutathione content was increased by both IL-4 and IFN-γ in comparison to untreated astrocytes (217 ± 9 μmol/g of protein at 2 h) (Fig. 5a). However, unlike IL-4, IFN-γ at a concentration of 100 ng/mL, resulted in a sustained increase in the glutathione pool size over a 24 h period (∼31% and ∼60% increase following 2 and 24 h of stimulation) (Fig. 5a). In contrast, after 2 h of stimulation, intracellular glutathione concentration increased by ∼43% (10 ng/mL IL-4), ∼35% (100 ng/mL IL-4), and ∼40% (10 ng/mL IFN-γ). After 24 h of stimulation, the increase in glutathione pool size was reduced to ∼4% (10 ng/mL IL-4), ∼15% (100 ng/mL IL-4), and ∼26% (10 ng/mL IFN-γ) (Fig. 5a). Increased biosynthesis of glutathione could represent an autocorrective metabolic response to enhanced intracellular production of ROS (Mosharov *et al.* 2000; Vitvitsky *et al.* 2003). To address this possibility, we examined the production of ROS in astrocytes after incubation with either $CD3^+$ T cells or with IFN-γ. Astrocytes produced ∼40% and ∼30% higher intracellular ROS upon incubation, with $CD3^+$ cells and IFN-γ, respectively, for 2 h (Fig. 5b).

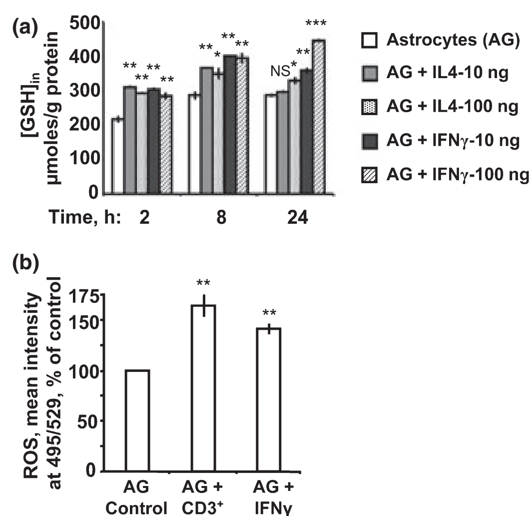


**Fig. 5** Dose-dependent effect of IL-4 and IFN-γ on intracellular glutathione synthesis and intracellular ROS generation in astrocytes. (a) Murine astrocytes were incubated alone (open bar), with 10 ng/mL IL-4 (gray bar) or 100 ng/mL IL-4 (dotted gray bar), 10 ng/mL IFN-γ (black bar), or 100 ng/mL IFN-γ (dotted black bar) for the indicated time points. The intracellular glutathione concentration was measured as described under Materials and methods. Panel (b) shows ROS levels in astrocytes in response to incubation with $CD3^+$ T cells or IFN-γ stimulation. Results show the mean ± SD of two independent experiments that were performed in duplicate. Statistical analysis using the Student's *t*-test revealed significant changes in intracellular glutathione concentration as a function of time and treatment with either cytokines versus the untreated control; *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$; ns, not significant.

### Cytokines stimulate lactate release by astrocytes

Lactate, a product of anaerobic glycolysis, is released by astrocytes and serves as a fuel molecule for neurons (Bouzier-Sore *et al.* 2002; Gladden 2004). We have assessed whether lactate secretion by astrocytes is stimulated by IL-4 and IFN-γ each added at a concentration of 20 or 100 ng/mL. (Fig. 6). A time-dependent increase in extracellular lactate concentration from 0.09 ± 0.05 mM at 2 h to 2.1 ± 0.04 mM at 24 h was observed in astrocyte cultures. The presence of cytokines markedly enhanced lactate secretion after 2 h with ∼900% and ∼500% increases being elicited by IL-4 and IFN-γ, respec-

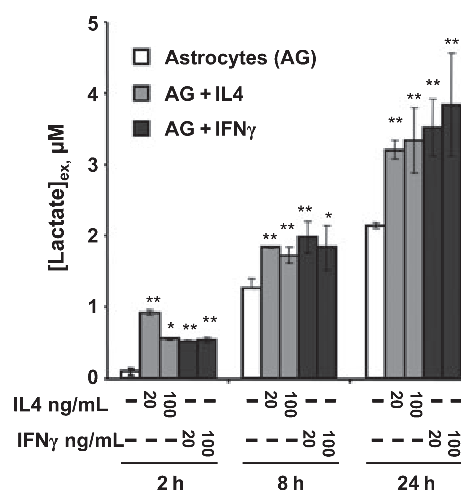


**Fig. 6** Cytokines influence lactate secretion by astrocytes. The culture media from murine astrocytes incubated alone (open bar), or with the indicated concentration of IL-4 (gray bar) or IFN-γ (black bar) were collected at the indicated time points and the concentration of lactate was analyzed as described under Materials and methods. The results show the mean ± SD and are representative of two independent experiments that were each performed in triplicates on different batches of cells. Statistical analysis using the Student's *t*-test revealed significant changes in lactate production by astrocytes as a function of time and treatment with either cytokines versus the untreated control; *$p < 0.01$; **$p < 0.001$; ns, not significant.

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tively. Upon longer incubation (24 h), a smaller ~55% increase in extracellular lactate concentration was observed in cytokine-treated cultures compared with untreated controls (Fig. 6).

### The neuroprotective effect of cytokine-primed astrocytes

Conditioned media from astrocytes primed with $CD3^+$ T cells were previously shown to protect neurons against *t*-BuOOH-induced oxidative stress (Garg *et al.* 2008) and the protection was associated with enhanced release of the neuroprotectants, cysteine and lactate triggered by a glutamate signaling pathway. As cytokines were found to stimulate the release of neuroprotective thiols and/or lactate from astrocytes (Figs 3 and 6), the effectiveness of astrocyte-conditioned media from these cultures in protecting neurons from apoptosis induced by oxidative stress was examined. Astrocytes were stimulated with 10 or 100 ng/mL each of IL-4 or IFN-γ for 10 h before conditioned media from various cell cultures were transferred onto neuronal monolayers in the presence of 200 μM *t*-BuOOH. The extent of apoptosis was assessed after 10–12 h by the TUNEL assay (Fig. 7). Astrocyte-conditioned medium alone afforded a modest but significant protection of neurons from apoptosis compared with fresh medium (51.3 ± 4.9% vs. 40.1 ± 3.8%) (Fig. 7a and c). However, when astrocyte-conditioned medium from cultures containing either 10 or 100 ng/mL IL-4 was added, a > 2-fold decrease in TUNEL positive cells was observed (40.1 ± 3.8% vs. 19 ± 5%). While a similar response was observed with conditioned medium from cultures treated with 10 ng/mL IFN-γ (40.1 ± 3.8% vs. 18.3 ± 2.9%), the protection was abolished when 100 ng/mL IFN-γ was used (40.1 ± 3.8% vs. 44.2 ± 9.1%) (Fig. 7a and c).

Primary neuronal cultures are usually contaminated with astrocytes, which may contribute to the neuronal protection seen under oxidative stress conditions. To eliminate the potential contribution of contaminating astrocytes in this assay, the above experiments were repeated with the transformed neuronal cell line, MES 21.2 (Fig. 7b). A similar neuroprotective effect was observed with conditioned medium derived from astrocytes primed with IL-4 (both concentrations), or IFN-γ (10 ng/mL). At higher concentrations, IFN-γ conferred lower protection on MES cells as also seen with primary neurons (Fig. 7b).

### Direct effect of cytokines on neurons under normoxic and oxidative stress conditions

As IFN-γ was shown to enhance amyloid-$\beta_{1-42}$-induced neuronal death (Bate *et al.* 2006), the response of oxidatively stressed neurons to the direct administration of cytokines was investigated. The presence of IL-4 at both low and high concentrations and or of IFN-γ at 10 ng/mL did not influence neuronal viability (Fig. 8a). In contrast, 100 ng/mL IFN-γ increased neuronal death by ~27% in comparison to the untreated control (Fig. 8a).

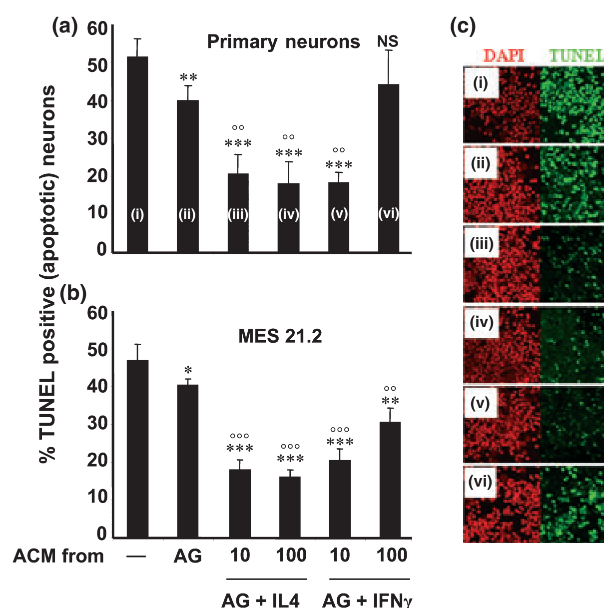


**Fig. 7** Th1 and Th2 cytokine-primed astrocytes are neuroprotective. Primary murine cortical neurons (a) or MES 21.2 cells (b) were treated with 200 μM *t*-BuOOH for 10 h in the presence of either fresh medium, or conditioned medium from astrocytes or conditioned medium from astrocytes treated with IL-4 or IFN-γ (10 and 100 ng/mL each). Bar graphs represent the mean ± SEM of apoptotic neuronal cells measured by TUNEL labeling as a percentage of total cells (labeled by Hoechst). The data were analyzed by counting at least 4000 cells (a) or 2000 cells (b) for each treatment in three (a) or two (b) independent experiments. Statistical analysis using the Student's *t*-test (paired, two-tailed distribution) was performed and individual *p* value revealed significant protection of neurons against *t*-BuOOH after exposure to conditioned medium from 10 ng/mL IFN-γ treated astrocytes but not with 100 ng/mL treated one. Conditioned medium from astrocytes exposed to either concentration of IL-4 was able to rescue the neurons; **P* < 0.01; **,°°*P* < 0.001; ***,°°°*P* < 0.0001; ns, not significant; * = (i) versus (ii)–(vi) and ° = (ii) versus (iii)–(vi). (c) Representative micrographs of apoptotic neurons are presented.

Next, the effect of direct exposure to cytokines on neuronal viability under normoxic conditions was examined. Both IFN-γ and IL-4 at lower concentrations increased neuronal apoptosis from the 5% level seen with untreated controls to 10% (Fig. 8b). A three- and five-fold increase in neuronal apoptosis was observed upon treatment of neuronal cultures with 100 ng/mL of IL-4 and IFN-γ, respectively (Fig. 8b).

## Discussion

The recruitment of infiltrating immune cells to the CNS is reported to contribute to secondary neurodegeneration (Popovich *et al.* 1996; Jones *et al.* 2002) but also to neuroprotection under acute injury or chronic neurodegenerative conditions (Hirschberg *et al.* 1998; Moalem *et al.*

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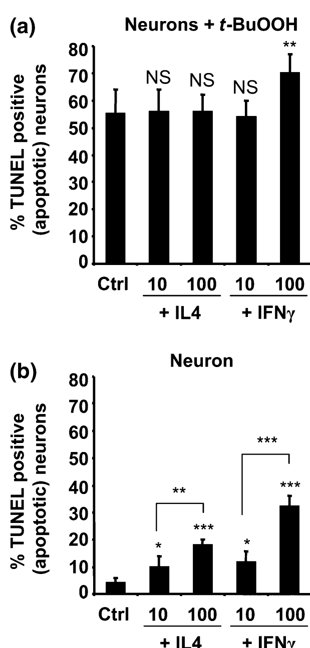


**Fig. 8** Neuronal response to cytokines in the presence or absence of *t*-BuOOH. Primary neurons were either untreated or incubated with 10 or 100 ng/mL of IL-4 or IFN-γ for 10 h at 37°C in the presence (a) or absence (b) of 200 μM *t*-BuOOH. Bar graphs represent the mean ± SEM of apoptotic neuronal cells measured by TUNEL labeling as a percentage of total cells (labeled by Hoechst). The data were analyzed by counting at least 2000 cells for each treatment from two independent experiments. Statistical analysis using the Student's *t*-test (paired, two tailed distribution) was performed and individual *p* value are shown; $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$; ns, not significant.

1999a,b). For example, the presence of IFN-γ was shown to be associated with neurodegeneration and tissue destruction (O'Garra *et al.* 1997; Wolf *et al.* 2002) but also to play an important role in neuroprotection (Kipnis *et al.* 2002b). These conflicting results emphasize the need for further research on the effects of T cell subtypes and their cytokines in influencing the outcome of CNS injury and motivated the present study.

Based on the results obtained in this study, we propose a model in which a controlled and well-timed immune response involving pro- and anti-inflammatory cytokines is predicted to be critically important for modulating a beneficial outcome following CNS injury (Fig. 9). Infiltration of IFN-γ producing (presumably Th1) cells into the CNS, is needed for preserving a key astroglial function that is compromised under the oxidative stress conditions typically associated with injury, namely, glutamate clearance (Fig. 1b). IFN-γ has the added effect of stimulating astrocytic secretion of thiols and lactate (Figs 3, 4, and 6), and represents another protective feature in the phenotypic modeling of astrocytes by T cells (Garg *et al.* 2008). Interestingly, co-culture of astrocytes with pan isolated and activated $CD3^+$ T cells (or exposure to IFN-γ) elicits higher intracellular ROS levels in astrocytes (Fig. 5b), which could be involved in signaling and may be a component of the innate immune response of astrocytes (Scumpia *et al.* 2005; Farina *et al.* 2007). Cells respond to an increase in ROS by up-regulating glutathione synthesis (Mosharov *et al.* 2000; Vitvitsky *et al.* 2003) and a similar response is suggested by the observation that astrocytes exposed to IFN-γ have higher intracellular glutathione levels (Fig. 5a).

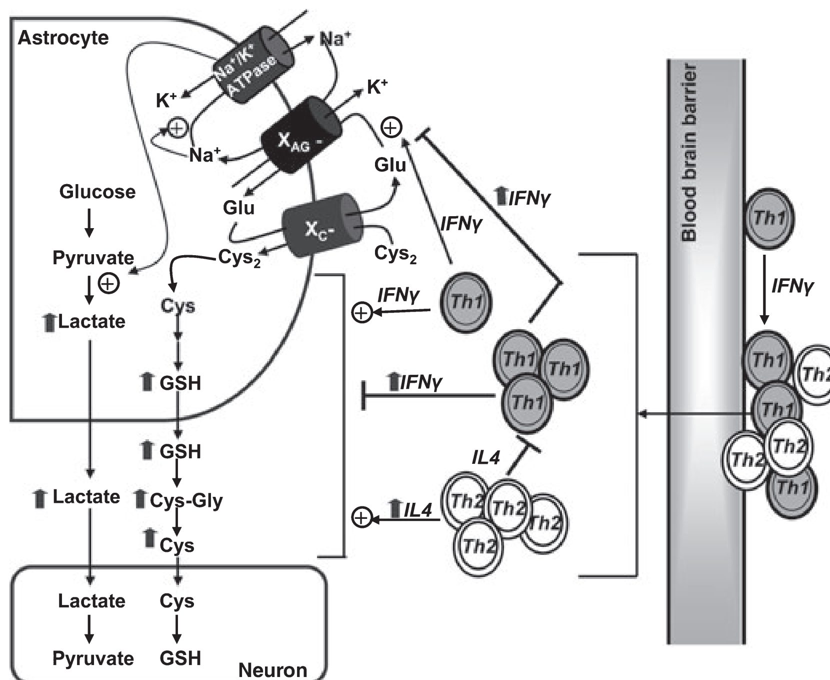


**Fig. 9** Proposed model for the role of a balanced Th1 and Th2 response in modulating neuroprotection by astrocytes. A low Th1 response (low IFN-γ production) is beneficial for both glutamate clearance and for enhancing thiol release from astrocytes. A Th2 response also enhances neuroprotectant release from astrocytes but does not preserve their glutamate clearance function. In the absence of a Th2 response, accumulation of high IFN-γ would adversely affect glutamate clearance and thiol release. Th2 cytokines restrict the proliferation of Th1 cells thereby limiting their noxious effects under conditions of continuous or unchecked stimulation. Thus, a balanced Th1 and Th2 response may afford the best neuroprotection.

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An overactive pro-inflammatory response is expected to be deleterious since at high concentrations, IFN-γ is significantly less effective at preserving astrocytic glutamate clearance and diminishes extracelluar cysteine levels. The extracellular thiols (glutathione, Cys-Gly, and cysteine) may be involved directly in neutralizing ROS whose concentrations are enhanced at sites of injury, or indirectly, by serving as a source of neuronal cysteine needed for biosynthesis of the antioxidant, glutathione. We find that while high IFN-γ levels diminish extracellular glutathione and Cys-Gly levels (Fig. 4a and b), the precursors of cysteine, the magnitude of inhibition is much greater on extracellular cysteine accumulation (Fig. 3a). This could result from inhibition of the dipeptidase that releases cysteine from Cys-Gly or from enhanced oxidation of cysteine to cystine when IFN-γ levels are high. Further studies will address whether IFN-γ is a prototype for how other pro-inflammatory cytokines, such as IL-12 and possibly also tumor necrosis factor-α affect astrocyte function, or whether it is unique in its ability to control astrocyte function.

The detrimental effects of an overly intense IFN-γ response may be avoided by IL-4, which boosts the neuroprotective effects on thiol and lactate secretion on the one hand and inhibits release of Th1 cytokines on the other (Fig. 9). Unlike IFN-γ, IL-4 enhances thiol and lactate release in a dose-dependent manner (Figs 3, 4, and 6) and does not restore glutamate uptake by oxidatively stressed astrocytes (Fig. 1d). Hence, an exclusive IL-4 (and presumably other anti-inflammatory cytokine) response may not be as effective in modulating a neuroprotective phenotype in astrocytes as build up of extracellular glutamate would cause excitotoxicity. In contrast, a balanced Th1 and Th2 response, by protecting both arms of the neuroprotective response in astrocytes, i.e. glutamate clearance and enhanced release of thiols and lactate, could play a critical role in stemming the spread of secondary degeneration. In addition to regulation of astrocytic redox state, IL-4 and other anti-inflammatory cytokines, such as IL-10, can affect astrogliosis, associated with CNS injury and can, thus, modulate astrocytic response to injury (Balasingam and Yong 1996; Woiciechowsky *et al.* 2004; Gorantla *et al.* 2007). In our *in vitro* system, detrimental effects of astrocytes on neuronal survival are not seen and hence it is possible that the effect of an IL-4-mediated neuroprotective response *in vivo* is substantially more robust than that observed *in vitro*.

Microglia are also believed to play a key role in post-injury degenerative and regenerative processes by virtue of their ability to reactivate infiltrating T cells (Ankeny and Popovich 2008). The interaction of microglia with neurons was recently shown to increase neuronal fibroblast growth factor-2 expression, which protects neurons against excitotoxicity (Figueiredo *et al.* 2008). Moreover, microglia are proposed to function as guardians of neuronal integrity (Butovsky *et al.* 2005; Shaked *et al.* 2005) because of their abundance at injury sites. Activation of microglia with IL-4 or IFN-γ was shown to induce oligodendrogenesis and neurogenesis, respectively (Butovsky *et al.* 2006). In contrast to microglia, the potential neuroprotective role of astrocytes elicited by their responses to pro- and anti-inflammatory cytokines has not been addressed. Our model for a controlled IFN-γ and IL-4-mediated activation of astrocytes for neuroprotection (Fig. 9) might also be relevant to the role of microglia at sites of injury and merits testing. It is important to note that the response of astrocytes to T cell-derived cytokines is likely to be influenced by the presence of microglia, which was not addressed in this study.

In summary, our results suggest that an uncontrolled boost of IFN-γ or IL-4 and presumably other pro- and anti-inflammatory cytokines might exacerbate the deleterious outcomes of CNS injury or chronic neurodegeneration. We propose that the timing of the response is likely to be important and substitution of an initial IFN-γ or other pro-inflammatory response by an anti-inflammatory one, such as IL-4, may eliminate a key protective effect of immune cells on astrocytes, i.e. preservation of their glutamate clearance function. Indeed, our data reveal that high levels of IL-4 can negate the protective effect of IFN-γ (Fig. 2) on glutamate clearance by oxidatively stressed astrocytes. Without this capability, astrocytes are unable not only to clear the excitotoxic neurotransmitter but also unable to release thiols. Therefore, substitution of an early Th1 by a Th2/Th3 response (Ishikawa *et al.* 2007) could potentially be as damaging as an overwhelming Th1 response. Our results suggest a resolution of the apparent dichotomy between the efficacy of boosting a Th1 (Kipnis *et al.* 2002b) versus a Th2/Th3 (Faria and Weiner 2006) response to CNS injury and suggest that mucosal tolerance could benefit severed neural tissues if induced after injury to allow for an initial Th1 wave that is counteracted by an induced Th2/Th3 response at a later time. The post-injury outcome of concomitant versus sequential intervention with Th1 and Th2 cytokines on shaping an astrocytic repair phenotype are not known and given the therapeutic relevance of this issue, merits further investigation.

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