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Astrocytes as antigen-presenting cells: expression of IL-12/IL-23

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Abstract

Interleukin-12 (IL-12, p70) a heterodimeric cytokine of p40 and p35 subunits, important for Th1-type immune responses, has been attributed a prominent role in multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). Recently, the related heterodimeric cytokine, IL-23, composed of the same p40 subunit as IL-12 and a unique p19 subunit, was shown to be involved in Th1 responses and EAE. We investigated whether astrocytes and microglia, CNS cells with antigen-presenting cell (APC) function can present antigen to myelin basic protein (MBP)-reactive T cells, and whether this presentation is blocked with antibodies against IL-12/IL-23p40. Interferon (IFN)-γ-treated APC induced proliferation of MBP-reactive T cells. Anti-IL-12/IL-23p40 antibodies blocked this proliferation. These results support and extend our previous observation that astrocytes and microglia produce IL-12/IL-23p40. Moreover, we show that stimulated astrocytes and microglia produce biologically active IL-12p70. Because IL-12 and IL-23 share p40, we wanted to determine whether astrocytes also express IL-12p35 and IL-23p19, as microglia were already shown to express them. Astrocytes expressed IL-12p35 mRNA constitutively, and IL-23 p19 after stimulation. Thus, astrocytes, under inflammatory conditions, express all subunits of IL-12/IL-23. Their ability to present antigen to encephalitogenic T cells can be blocked by neutralizing anti-IL-12/IL-23p40 antibodies.

Keywords: antigen presentation, astrocyte, experimental autoimmune encephalomyelitis, interleukin-12, microglia.
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Astrocytes represent the most abundant glial cell type and have important roles in the central nervous system (CNS): they provide physical and metabolic support to neurons and participate in the formation and maintenance of the blood–brain barrier (Minagar *et al*. 2002). They also produce neurotrophic and neuroprotective factors, guide neurons, regulate the extracellular pH and potassium levels, and participate in repair processes within the CNS (Minagar *et al*. 2002). An important, albeit less well-characterized, immune function has also been described for astrocytes (Dong and Benveniste 2001). They release pro-inflammatory cytokines, including interleukin (IL)-1, IL-6, and tumour necrosis factor-α (TNF-α) in response to a variety of stimuli (Benveniste 1992; Dong and Benveniste 2001). They also have been implicated in antigen presentation in the CNS (Fontana *et al*. 1984). The role of the microglia, the resident macrophages of the CNS, in antigen presentation, has been well established (Frei *et al*. 1987, 1988; Minagar *et al*.

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Abbreviations used: Ab, antibody; APC, antigen-presenting cells; EAE, experimental autoimmune encephalomyelitis; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; IL, interleukin; IFN, interferon; LPS, lipopolysaccharide; mAb, monoclonal antibody; MBP, myelin basic protein; MHC, major histocompatibility complex; OVA, ovalbumin; RIA, radioimmunoassay; TCR, T cell receptor.

2002), but the role of astrocytes has been more controversial. Several studies indicate that, unlike microglia, which are professional antigen-presenting cells (APC), astrocytes require activation, including induction of major histocompatibility (MHC) class I and II molecules by interferon (IFN)-γ, for presentation of antigen to CD8+ or CD4+ T cells, respectively (Fierz *et al*. 1985; Fontana *et al*. 1986). Furthermore, microglia are also much more efficient APC when pre-stimulated with IFN-γ (Frei *et al*. 1987). Astrocytes are therefore classified as non-professional APC. The factor determining the professional APC status for CD4+ cells, important effector cells in multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), is the MHC class II transactivator (CIITA). There are several different, cell-type specific promoters: pI and pIII are utilized in the professional APC. The type IV promoter (pIV) is utilized in astrocytes (Dong *et al*. 1999; Stuve *et al*. 2002).

Some authors state that, perhaps because of lack of expression of co-stimulatory molecules, astrocytes may deliver an inhibitory or tolerogenic signal (Meinl *et al*. 1994; Aloisi *et al*. 2000). However, it has been shown that, when astrocytes as APC are in a ratio close to 1 : 1 to T cells, they are fully competent APC. That study also demonstrated that astrocytes can also express B7 co-stimulatory molecules (Nikcevich *et al*. 1997).

MS is a neurological disease characterized by inflammation and demyelination in the CNS. It bears numerous similarities with EAE, a CNS inflammatory disease that can be induced by active immunization with myelin antigens in complete Freund's adjuvant (CFA) or by transfer of T cells reactive against these antigens to naïve animals. The encephalitogenic T cells typically exhibit the Th1 phenotype, characterized by production of IFN-γ and TNF (Constantinescu *et al*. 1998a).

IL-12 is a heterodimeric cytokine of two subunits, a largely constitutively expressed p35 and a highly inducible p40 subunit produced in excess over the biologically active heterodimer (p70) upon stimulation. There is generally a good correlation between the levels of p40 and p70. IL-12 is produced by antigen-presenting cells and phagocytic cells, and has been shown to be important for the development of Th1-type immune responses (Trinchieri 1998). Antibodies directed against IL-12p40 (the unit shared with IL-23, see below) prevent the development of EAE in a monophasic model (Leonard *et al*. 1995; Constantinescu *et al*. 2001). In addition, these antibodies prevent both spontaneous and superantigen-induced relapses (Constantinescu *et al*. 1998b). Moreover, exogenous IL-12 exacerbates EAE by inducing relapses and enhancing the destructive pathology in both mouse and rat models (Smith *et al*. 1997; Constantinescu *et al*. 1998b). IL-12 mRNA (both for p40 and p35 subunits) was demonstrated in the CNS mainly at the early acute stage of paralysis in EAE, followed by rapid down-regulation in remission stages (Issazadeh *et al*. 1995; Bright *et al*. 1998), similar to its presence being restricted to the acute lesions in the CNS of MS patients (Windhagen *et al*. 1995).

Recently, an IL-12 related cytokine, IL-23, was discovered computationally (Oppmann *et al*. 2000). IL-23 has the same p40 subunit as IL-12 and a unique p19 subunit. IL-23 is also important in Th1-type responses and IL-23, rather than IL-12, appears to be the main responsible cytokine for EAE (Gran *et al*. 2002; Cua *et al*. 2003). As a result of enhanced severity of EAE in IL-12p35-deficient mice (Gran *et al*. 2002), IL-12 is suggested even to have a protective role (Gran *et al*. 2004), as in another autoimmune inflammatory condition, a model of arthritis (Murphy *et al*. 2003).

A model has been proposed whereby IL-12 is essential for T-cell priming and Th1 differentiation, while IL-23 is critical for established T-cell mediated inflammation, via its effects on memory T cells. However, regardless of the relative contribution of each cytokine to the various stages of EAE, because IL-12 and IL-23 share the p40 subunit, targeting IL-12/IL-23p40, for example with neutralizing antibodies, may be an effective method to eliminate the most significant CNS inflammatory processes in EAE and possibly MS.

Because the presence of IL-12/IL-23p40 mRNA in the active stages of MS or EAE may imply a role for IL-12/IL-23p40 in antigen presentation in the CNS, we wanted to determine whether the professional and non-professional APC of the CNS, microglia and astrocytes, can stimulate neuroantigen-reactive T cells in an IL-12/IL-23-dependent manner. We show that antibodies against IL-12/IL-23p40 inhibit antigen presentation by both astrocytes and microglia. We demonstrate the ability of CNS APC to produce the biologically active heterodimer, IL-12p70. We also show expression of the p35 subunit of IL-12 and of p19 subunit of IL-23 by astrocytes.

Materials and methods

Animals

Newborn (day 1–3) (PL/J × SJL/J) F1 mice (Jackson Laboratories, Bar Harbor, ME, USA) were used for isolation of CNS astrocytes and microglia.

Reagents

Myelin basic protein (MBP), prepared from guinea pig spinal cord (Rockland, Gilberstville, PA, USA) according to the method of Deibler *et al*. (1972) was lyophilized and stored at −20°C until use. Murine recombinant IFN-γ was purchased from PharMingen (San Diego, CA, USA). The following monoclonal anti-mouse IL-12/IL-23p40 antibodies, each of which recognizes a distinct epitope on IL-12/IL-23p40, were used: C17.8 (rat IgG2a) was used for antibody neutralization in antigen presentation assays; C15.1 (rat IgG1) and C15.6 (rat IgG1) were used in a two-site radioimmunoassay (RIA) as previously described (Wysocka *et al*. 1995). LPS was from Sigma (St Louis, MO, USA). IFN-γ and IL-1 were from R & D Biosystems, Abingdon, UK).

Isolation of astrocytes and microglia

Astrocytes and microglia were prepared as described (Constantinescu *et al*. 1996). Briefly, brains were sequentially dis-aggregated through 100- and 70-μm nylon sieves (Falcon, Becton-Dickinson, San Jose, CA, USA), seeded in culture flasks with Dulbeco's modified Eagle's medium (DMEM) plus 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 mg/mL) with 20% fetal calf serum (FCS); medium was changed to 10% FCS (day 4–5) and 5% FCS (day 9–10). When cultures reached confluence (day 15–19) astrocytes and microglia were separated through differential adherence after 1 h of vigorous shaking in a shaker/incubator. The identity of separated cells was confirmed by indirect immunofluorescence with anti-glial fibrillary acidic protein (GFAP) antibody (Sigma) for astrocytes and either Mac-1 antibody (PharMingen) or non-specific esterase (Sigma). Microglia cultures were >97% positive for the microglial cell marker; astrocyte cultures were consistently >95% positive for GFAP. In some antigen presentation experiments, further purification was accomplished through panning of microglia. Panning was as follows: astrocyte-rich cultures were incubated on plates pre-coated with rat anti-mouse FcγR monoclonal antibody (mAb) 2.4G2 (supernatant of hybridoma from ATCC, Rockville, MD, USA) for 2 h at room temperature (20°C). The cells obtained after panning were consistently >98% GFAP+. Consistently less than 1% Mac-1+ cells were present in the culture.

Splenic macrophages were obtained by removing the spleens and passing them through a stainless steel mesh. Red blood cells were removed by hypotonic lysis in NH_4CL-containing buffer. Homogenized single cell suspensions were re-suspended in RPMI containing 5% fetal bovine serum and antibiotics and allowed to adhere for 45 min at 37°C in an incubator with 5% CO_2. RAW murine macrophage cell line was used as positive control in some experiments.

Proliferation assays

The LV-4 T-cell line is previously described (Constantinescu 1997) MBP-reactive murine Th1 encephalitogenic cell line generated from (PL/J × SJL/J) F1 mice. Ovalbumin (OVA; Sigma) was used as negative control. APC were plated on 96-well plates at 1×10^6/mL, irradiated (3000 cGy) and stimulated with IFN-γ 100 U/mL for 72 h. In view of the findings of Nikcevich *et al*. (1997) all antigen presentation assays shown here were performed at an APC : T-cell ratio of 1 : 1.

LPS stimulation was with 1 μg/mL for the last 24 h of IFN-γ stimulation. LV-4 cells were added at 1×10^6/mL after 72 h of IFN-γ stimulation for an additional 48 h. Antigens were added in the concentration of 10 μg/mL simultaneously with the T cells. During the last 12 h of stimulation, the cells were pulsed with ^{3}H-thymidine. Cells were harvested and ^{3}H-thymidine incorporation was measured in a β-scintillation counter (Beckman LS8000). The stimulation index was read as the ratio of thymidine incorporation in the presence and absence of the stimulus in the cell-culture condition.

RT-PCR

RNA was extracted from cultured cells using the GITC method, reverse-transcribed with Maloney mouse leukaemia virus reverse transcriptase (M-MLV RT, United States Biochemical). For p40 and p35, cDNA was amplified using p40-specific or p35-specific oligonucleotide primers with published sequences (Murphy *et al*. 1994) (synthesized in the Cell Center, University of Pennsylvania or at MWG Biotech AG, Germany). Primers for the HPRT gene were used as positive controls. For p19, we used the following primers: forward 5′-TGCTGGATTGCAGAGCAGTAA-3′ (Parham *et al*. 2002); reverse 5′-AGTCCTTGTGGGTCACAACC-3′ (expected product length, 248 bp). PCR was performed under the following conditions: initial denaturation 94°C for 5 min; followed by 35 cycles of: denaturation 94°C for 1 min; annealing 55°C for 1° min; extension 72°C for 1 min; followed by a final extension at 72°C for 1 min.

Measurement of IL-12/IL-23p40 and IL-12p70 protein

The levels of IL-12/IL-23p40 were measured in astrocytes and microglia supernatants using a two-site RIA as previously described (Wysocka *et al*. 1995). C15.1 (5 μg/mL) was used as capture antibody and C15.6 (0.1 mg/mL) as detecting antibody. IL-12p70 was measured using an ELISA (DuoSet ELISA Development kit DY419 purchased from R & D Biosystems, using part 840131 at 4 μg/mL as capture antibody and part 840132 at 0.3 μg/mL as detecting antibody).

We also performed bioassays for IL-12p70 based on its ability to induce IFN-γ in BALB/c mouse splenocytes (Gately and Chizzonite 1992).

Results

Lymphocyte proliferation

We utilized astrocytes, microglia and spleen macrophages as APC for an MBP-reactive encephalitogenic T-cell line, LV-4. Microglia from neonatal (PLJ/J × SJL/J) F1 mice were harvested, purified, irradiated (3000 cGy) and stimulated with IFN-γ for 72 h for optimal MHC class II induction as described (Frei *et al*. 1987). With microglia as APC, the MBP-reactive T cells responded to antigenic stimulation by proliferation as shown by ^{3}H-thymidine incorporation. The proliferation was significantly blocked by addition of anti-IL-12/IL-23p40 mAb C17.8 to the stimulation culture, while an isotype matched control antibody (Ab) (IgG2a) had no significant effect.

These findings suggest that antigen presentation by microglia is IL-12/IL-23 dependent; moreover, they suggest that the T-cell–microglia interaction results in IL-12/IL-23 induction, as IL-12/IL-23 neutralization blocked T-cell proliferation. Because LPS is a strong stimulus for IL-12 production [and possibly also, but likely less, potent for IL-23 production (Begum *et al*. 2004)] we stimulated microglia in the last 24 h of IFN-γ stimulation additionally with LPS, to determine the effects on antigen presentation and its IL-12/IL-23 dependence. Stimulation under these conditions induced a dramatic increase in T-cell proliferation in response to MBP, which was also in great part IL-12/IL-23p40 dependent: anti-IL-12/IL-23p40 antibody significantly suppressed this proliferation, while the control antibody had no significant effect (Fig. 1a).

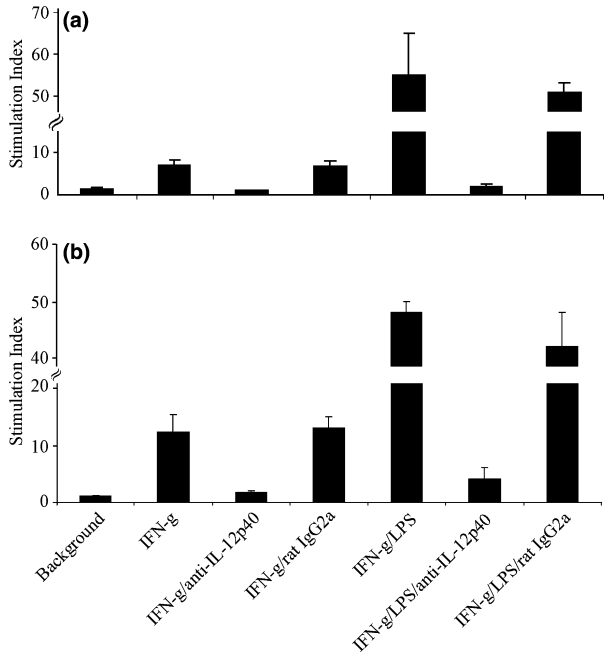

Fig. 1 (a) Antigen presentation to MBP-reactive T-cell line LV-4 by IFN-γ-pretreated microglia. Microglia were harvested as described in Materials and methods, plated on 96-well plates and irradiated (3000 cGy). IFN-γ stimulation was with 100 U/mL for 72 h. LPS stimulation was with 1 μg/mL for the last 24 h of IFN-γ stimulation. Microglial cells and MBP-reactive T cells (10^5) were used in these assays. [^{3}H]-thymidine was added to the last 16 h of the stimulation and proliferation was measured by [^{3}H]-thymidine incorporation. Anti-IL-12p40 mAb or control IgG2a (50 μg/mL) were added at the start of culture. Results are expressed as means and range of duplicate cultures. Similar results were obtained in two additional experiments. (b) Antigen presentation to MBP-reactive T-cell line LV-4 by IFN-γ-pretreated astrocytes. Astrocytes were harvested as described in Materials and methods, plated on 96-well plates and irradiated (3000 cGy). Experimental conditions including T-cell : APC ratio were as in (a). Results are expressed as means and range of duplicate cultures. Similar results were obtained in two additional experiments.

Because microglia are CNS macrophages, which have previously been shown to produce both the p40 and the p35 subunit of IL-12 as well as the p19 subunit of IL-23, it is not altogether surprising that they are capable of antigen presentation in an IL-12/IL-23-dependent manner. Spleen macrophages under identical stimulation conditions, used as APC, showed similar results (data not shown).

MBP-reactive T cells stimulated with OVA did not proliferate; likewise, these T cells stimulated with MBP, LPS, or both in the absence of APC, did not proliferate (data not shown).

IFN-γ treated astrocytes were similarly capable of inducing proliferation of MBP-reactive T cells (Fig. 1b), confirming previous results (Fierz *et al.* 1985). The addition of IL-12-inducer LPS also enhanced T-cell proliferation dramatically. T-cell proliferation results were similar when equivalent numbers of astrocytes and microglia were used as APC (Fig. 1b).

For both microglia and astrocytes, optimal suppression of proliferation was obtained with the anti-IL-12/IL-23p40 antibody added immediately after the T cells and antigens, for a total time of 48 h. Partial suppression was also noted with the antibody added 24 h after the T cells (data not shown).

The fact that in the astrocytes–T-cell interaction antigen-induced T-cell proliferation was IL-12/IL-23p40-dependent also suggests that IL-12/IL-23p40 is induced by this interaction.

Astrocytes produce p40 and p70

The above findings suggest that CNS APC, microglia and astrocytes, can produce IL-12/IL-23p40. Previously, we showed expression of IL-12/IL-23p40 mRNA by LPS-stimulated microglia and astrocytes, as well as IL-12/IL-23p40 protein based on a sensitive RIA (Constantinescu *et al.* 1996). The previous RT-PCR findings were repeated in additional astrocyte and microglia cultures and shown to be consistent. Direct sequencing of the PCR product identified it unequivocally as murine IL-12/IL-23p40. Because LPS stimulation can result in astrocyte proliferation, but little or no microglia proliferation in pure culture (in the absence of microglia/macrophage growth factors such as GM-CSF or M-CSF which normally would be supplied by astrocytes in mixed cultures), we measured IL-12/IL-23p40 in cultures highly enriched for microglia or astrocytes and analyzed the results normalized for cell number (based on count of nuclei). Significant amounts of IL-12/IL-23p40 were measured in both cell types by RIA after LPS stimulation (Fig. 2).

We also measured IL-12p70 using an ELISA (Fig. 3). Both microglia and astrocytes were shown to produce IL-12p70 after LPS stimulation.

Moreover, we detected biologically active IL-12 by bioassay based on the ability of supernatants to induce IFN-γ production by BALB/c splenocytes (Fig. 4). We did not detect IL-12/IL-23p40 or IL-12p70 in astrocytes or microglia cultures stimulated with IFN-γ alone.

Astrocytes express IL-12p35 and IL-23p19

The ability of astrocytes to express and produce IL-12/IL-23p40 may signify that these glial cells are a potential source of IL-12 and/or IL-23. Alternatively, astrocytes may only express IL-12/IL-23p40, as has been proposed by some authors (Aloisi *et al.* 1997). Therefore, it was interesting to determine whether astrocytes can also express the other components of the IL-12/23 group of cytokines.

We performed RT-PCR on highly purified populations of astrocytes (> 98% GFAP+ obtained by eliminating the microglia through the panning method described above) and performed RT-PCR to look for IL-12p35 mRNA.

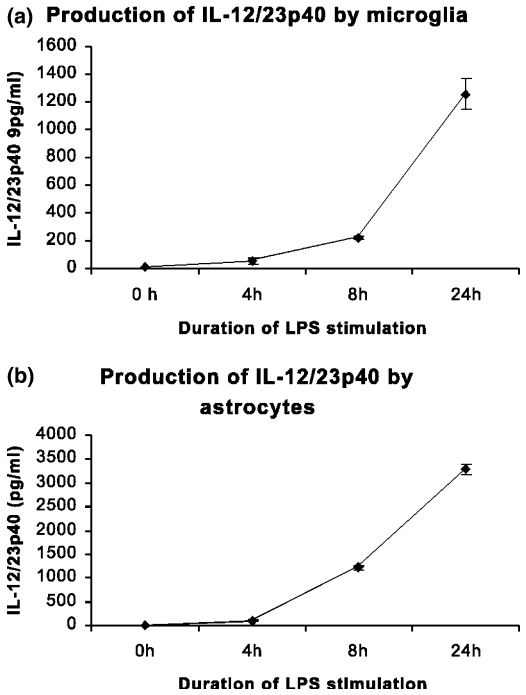

Fig. 2 Production of IL-12p40 by cultured microglia (a) or astrocytes (b) stimulated with LPS for the designated time intervals. The results are corrected for cell count at the end of the culture period. Results are shown as mean and standard deviations of three separate experiments.

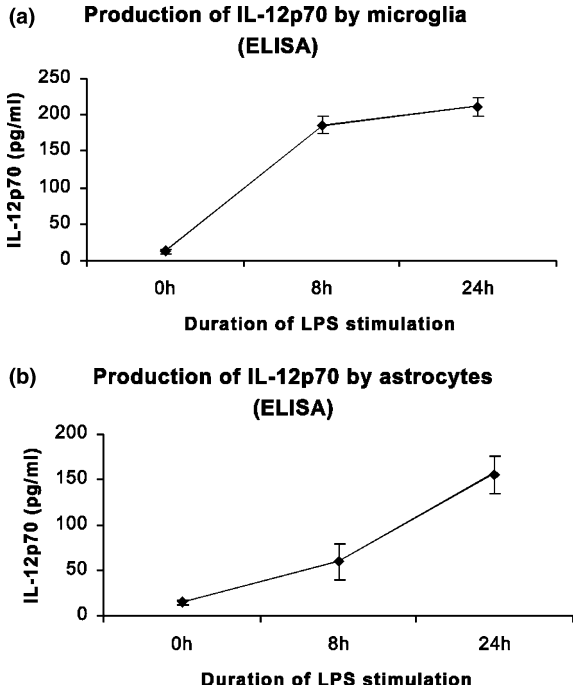

Fig. 3 Production of IL-12p70 by cultured microglia (a) or astrocytes (b) stimulated with LPS for the designated time intervals. The results are corrected for cell count at the end of the culture period. Results are shown as mean and ranges of two separate experiments. IL-12p70 was measured by ELISA.

Consistent with the existing data that IL-12p35 is largely expressed constitutively at low levels in a wide range of cell types, we detected IL-12p35, both in stimulated and in unstimulated astrocyte cultures (Fig. 5).

We also investigated the ability of astrocytes to express IL-23p19. Unstimulated astrocytes did not express p19; however, IFN-γ– and IL-1β-stimulated astrocytes were found to express p19 (Fig. 6). The identity of the weakly positive band was unequivocally identified as p19 by direct sequencing. RT-PCR performed on RAW macrophages yielded a unique positive band identified as p19 (data not shown). The experiments were repeated with a different pair of primers and also in neonatal astrocytes from SJL/J and C57/Bl6 mice with similar results. We also sequenced the 500 bp band (Fig. 6) consistently and specifically found in astrocyte cultures using two different pairs of primers. It has extensive sequence identity with p19 and is identical to a transcript found in neonatal mouse brain (AK043894 in GenBank mouse mRNAs; Okazaki *et al*. 2002). However, the purified 248 bp PCR product is clearly IL-23p19.

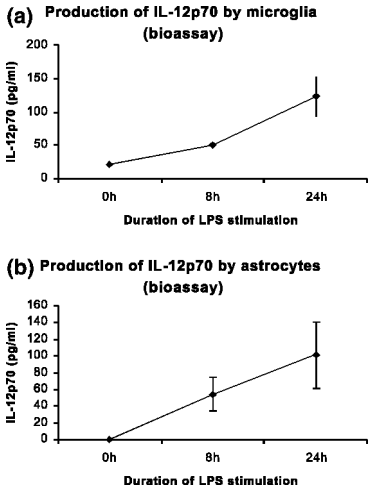

Fig. 4 Production of biologically active IL-12p70 as detected by bioassay by murine microglia (a) or astrocytes (b) stimulated for the indicated length of time. Results are expressed as means and ranges of two separate experiments. The results are corrected for the cell count at the end of the culture period.

Discussion

Previous studies have shown that IL-12/IL-23p40 mRNA is present in the CNS in acute phases of EAE, consistent with its detection in active lesions in MS brain. In a relapsing EAE model in (PL/J × SJL/J) F1 mice, the same strain used in most of these experiments, we have shown that the acute

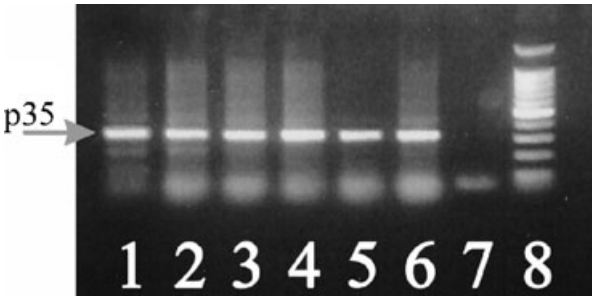

Fig. 5 Expression of IL-12p35 by purified mouse astrocytes unstimulated or stimulated as follows: 1, control (no stimulation); 2, IL-1β (10 ng mL)/IFN-γ (100 U/mL) 6 h; 3, LPS (1 μg/mL)/IFN-γ; 4, control; 5, IL-1β/IFN-γ; 24 h; 6, LPS/IFN-γ 24 h; 7, no RT control; 8, 100 bp molecular weight marker.

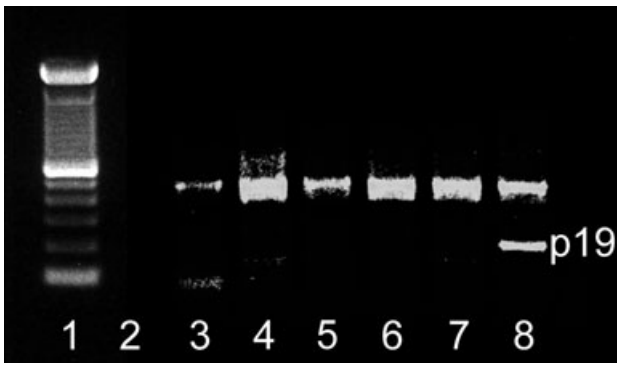

Fig. 6 Expression of IL-23p19 by purified mouse astrocytes unstimulated or stimulated as follows: 1, molecular weight marker; 2, gap; 3, control (no stimulation); 4, LPS; 5, IFN-γ; 6, IL-1β; 7, LPS/IFN-γ; 8, IL-1β/IFN-γ. Stimulus doses are as in Fig. 5 legend.

onset and the relapses are dependent on IL-12/IL-23p40 (Constantinescu *et al*. 1998b). Moreover, our unpublished results show IL-12/IL-23p40 mRNA in CNS of mice detectable only during the relapses but not in the remission stages. These observations suggest that CNS-derived IL-12/IL-23 may be involved in relapses.

Antigen presentation within the CNS is likely to represent an important phenomenon in the generation of relapses. During the initial acute phase of EAE, it is thought that new neuroantigen epitopes are being exposed to immune cells following the initial CNS inflammation and damage, and new sets of neuroantigen-reactive T cells develop (McRae *et al*. 1995; Vanderlugt and Miller 1996; Tuohy *et al*. 1998). The implication of this mechanism is that the local CNS environment facilitates the emergence of these T cells. Indeed CNS APC such as microglia and astrocytes under inflammatory conditions can express MHC class II molecules and co-stimulatory molecules capable of inducing T-cell activation. Moreover, because the new sets of T cells generated during epitope spreading are also of Th1 phenotype, it is implied that the CNS milieu is capable of directing the response toward a Th1 type. As IL-12 is pivotal to Th1 differentiation and development, and emerging evidence shows a role for IL-23 in T-cell responses resembling Th1, in particular in memory T cells, it is implied that IL-12 and/or IL-23 is being produced in the CNS. Here, we show that the two main potential APC within the CNS both are capable of producing biologically active IL-12. We also show that astrocytes are capable of expressing all the components of IL-12 and IL-23. Previous data showed that microglia also express p35 (Aloisi *et al*. 1997; Stalder *et al*. 1997) and p19 (Li *et al*. 2003). Whether these *in vitro* findings have *in vivo* relevance remains to be determined, as there are conflicting reports on the ability of astrocytes to effectively present antigen *in vivo* (Myers *et al*. 1993; Horwitz *et al*. 1999). One likely possibility is that microglia are more effective at triggering immune responses, while astrocytes may have a more important role in promoting and shaping ongoing responses.

Efficient activation of CD4 T cells requires two signals: the interaction between the TCR and the antigenic peptide presented by APC in the context of MHC class II, and additional co-stimulatory interactions, an important one being that between the B7 co-stimulatory molecule and the CD28 antigen on the surface of the T cell. MHC class II is not constitutively expressed on non-professional APC such as astrocytes, but can be up-regulated following IFN-γ treatment (Fierz *et al*. 1985). Likewise, significant MHC class II up-regulation occurs following IFN-γ treatment of microglia (Frei *et al*. 1987). With regard to the expression of B7 molecules, this has been documented on microglia (De Simone *et al*. 1995) (Menendez Iglesias *et al*. 1997). Studies investigating the expression of B7 co-stimulatory molecules on astrocytes have yielded conflicting results. Several studies failed to identify B7 expression on astrocytes (Aloisi *et al*. 1995; Cross and Ku 2000).

Because TCR ligation in the absence of co-stimulation leads to inefficient activation and anergy induction, the absence of B7 on astrocytes has been used as an argument to support the concept of astrocytes as inefficient APC, and to attribute them a protective, tolerizing role in CNS immune responses (Meinl *et al*. 1994). However, a number of investigations of highly pure murine astrocyte cultures demonstrated the inducibility of B7-1 and B7-2 on astrocytes (Nikcevich *et al*. 1997; Tan *et al*. 1998; Soos *et al*. 1999).

In a direct comparison of microglia and astrocytes as APC to OVA-reactive T cells from mice transgenic for an OVA-specific TCR, it was concluded that microglia are more efficient APC than astrocytes to Th1 cells but not to Th2 cells (Aloisi *et al*. 1998). Our present study shows similar efficiencies of antigen presentation by microglia and astrocytes to our Th1 cell line. The explanations for this discrepancy may be multiple. Differences in the experimental conditions include the use of a neuroantigen, MBP, in

contrast to OVA peptide as used in the study of Aloisi *et al*. (1998). The APC and T cells used in that study were from mice on a BALB/c background, known to be resistant to EAE. The OVA-reactive T cells were previously polarized during the culture by cytokine manipulation, IL-12 and anti-IL-4, to induce Th1 cells and IL-4 and anti-IL-12 for Th2 cells. The polarity was, however, relative, based on intracellular cytokine expression: the Th1 cells were 60% IFN-γ+ and the Th2 cells 44% IL-4+. Our long-term MBP-reactive line was generated by repeated stimulation with antigen without additional cytokine manipulation and it consistently produced large amounts of IFN-γ. Lastly, we pre-stimulated both cell types with IFN-γ for 72 h, and in some experiments with LPS (conditions found in preliminary experiments to be optimal for induction of T-cell proliferation), in contrast to shorter pre-stimulations used by Aloisi *et al*. (1998).

It is remarkable that, in these experiments, astrocytes responded so vigorously to LPS, with regard to induction of T-cell proliferation and IL-12/IL-23 p40 induction, although not IL-23 p19 induction. There are conflicting results in the literature with regard to the ability of astrocytes to respond to LPS, an ability related to whether astrocytes express toll-like receptors or not. Some studies have not identified toll-like receptors on astrocytes (Lehnardt *et al*. 2002), while others have found astrocytes to express such LPS-recognition structures (Bowman *et al*. 2003). These conflicting results may reflect strain- and species-specific differences, and may underlie differential susceptibility to infection or inflammatory autoimmunity (Alleva *et al*. 1998).

It is very unlikely that the IL-12/IL-23p40-dependent effects on astrocyte cultures, as shown in this paper, are as a result of contamination of cultures by non-astrocytic cells such as microglia, shown in this study also to produce IL-12. Studies using pure microglia cultures in numbers corresponding to maximal numbers of GFAP– cells in astrocyte cultures failed to reveal detectable IL-12 under stimulation conditions used in these experiments (data not shown). In addition, microglia in numbers corresponding to maximum concentration of GFAP– cells in astrocyte cultures (> 98% GFAP+) after panning for microglia were unable to induce significant proliferation of MBP-reactive T cells (data not shown).

We found a great degree of similarity between the results of IL-12p70 ELISA and IL-12p70 bioassay. The bioassay we used was based on the ability of IL-12 to induce IFN-γ in BALB/c mouse splenocytes. The first step in the assay was to place the supernatants in plates covered with anti-IL-12/IL-23p40 antibody. Other IL-12 bioassays using the same anti-IL-12/IL-23 antibodies are based on induction of splenocyte proliferation. Potentially, these assays may also detect IL-23 because the antibody used is against the p40 subunit. As current knowledge suggests that IL-12 is likely to be more effective in IFN-γ induction and IL-23 in stimulating proliferation, an IFN-γ-based bioassay is more likely to detect more IL-12 than IL-23. Murine IL-23 appears to be a modest inducer of IFN-γ compared with IL-12, while more capable of inducing IL-17 (Langrish *et al*. 2005). Indeed, the comparability of the bioassay results to those of the IL-12p70 ELISA indicate that, in fact, our bioassay largely measured IL-12.

The ability of astrocytes to express both components of IL-12 as shown in this study confirms several previous reports, but also contradicts other reports. As reviewed by Shrikant and Benveniste (1996), several differences in experimental conditions can explain differences between results aimed at determining cytokine production and antigen-presentation properties. We have mentioned differences in the antigen used, pre-stimulation and strain. In addition, the APC : T-cell ratio appeared to be essential in experiments showing B7-dependent T-cell proliferation with astrocytes as APC (Nikcevich *et al*. 1997). Other variables may also be important, including isolation of cells from adult versus neonatal brain, species differences, isolation conditions (e.g. trypsin use). Even the region of the brain from which cells are obtained may differentially influence astrocyte function (Shrikant and Benveniste 1996).

These results show that exposure of microglia and astrocytes to *in vitro* conditions simulating an inflammatory environment can stimulate neuroantigen-reactive T cells and can induce IL-12/IL-23 components. It has previously been shown that microglia express these components (Li *et al*. 2003), and other studies have shown astrocyte p35 expression (Stalder *et al*. 1997) while others have failed to find it (Aloisi *et al*. 1997). The demonstration of p19 in astrocytes is a novel finding. The specific expression of p19 following IL-1/IFN-γ stimulation may be explained by the fact that currently many of the potential stimuli for induction of the recently discovered pro-inflammatory cytokine, IL-23, are not well known and may differ from IL-12-inducing stimuli. In addition, p35 is constitutively expressed, while p19 is inducible.

Our finding makes astrocytes, to our knowledge, only the second non-tumoral, non-hematopoietic cell type to express both IL-12 components, the other cell type being keratinocytes (Aragane *et al*. 1994; Muller *et al*. 1994). Regarding expression of p19 in non-hematopoietic tissue, it has been reported in human intestinal epithelium (Maaser *et al*. 2004).

Our findings imply that efficient Th1 or Th1-like responses driven by IL-12 or IL-23 can be generated and perpetuated in the CNS. IL-12 plays a major role in the differentiation of T cells toward the Th1 phenotype. In contrast to naïve T cells, proliferation of differentiated Th1 cells requires the co-operation of membrane-bound co-stimulatory molecules, in particular B7, with IL-12, which in this aspect behaves as a soluble co-stimulatory molecule itself (Murphy *et al*. 1994). In addition, IL-12 also serves to enhance T-cell expression of additional co-stimulatory molecules important for Th1 responses, such as CD40L (Peng *et al*. 1998; Zhang *et al*. 2001). Neither IL-12 nor IL-23 increased expression of

co-stimulatory molecules in murine APC after exposure of up to 48 h. However, exposure to both cytokines enhanced antigen presentation function, and the most significant effect was observed when they were used in combination (Belladonna *et al*. 2002). Increasingly, the role of IL-23 is being recognized for proliferation of memory T cells, explaining the resistance to EAE of IL-23p19 knockout mice. Because, putting together our current results with previous findings, both astrocytes and microglia are capable of expression of both B7 co-stimulatory molecule family and IL-12/IL-23 component family, these studies indicate that both the astroglial and the microglial components of the CNS have the necessary immunological tools to initiate and perpetuate Th1-type responses to neuroantigens.

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