Soluble Tumor Necrosis Factor Receptor Inhibits Interleukin 12 Production by Stimulated Human Adult Microglial Cells In Vitro

Burkhard Becher, Vincent Dodelet, Valerie Fedorowicz, and Jack P. Antel
Neuroimmunology Unit, Montréal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montréal, QC H3A 2B4 Canada

Abstract

IL-12 is a cytokine detected in active lesions in multiple sclerosis (MS) and promotes the acquisition of a Th1 cytokine profile by CD4$^+$ T cells. Autoreactive T cells recovered from the central nervous system of animals with experimental autoimmune encephalomyelitis (EAE), a disease model for MS, display this phenotype. We demonstrate that human central nervous system–derived microglia, but not astroglia, can produced IL-12 in vitro. Under basal culture conditions, human adult microglia do not express detectable levels of IL-12, although these cells show some degree of activation as assessed by expression of the immunoregulatory surface molecules HLA-DR and B7 as well as low levels of TNF-α mRNA. Following activation with LPS, IL-12 p40 mRNA and p70 protein can be readily detected. IL-12 production is preceded by TNF-α production and is inhibited by recombinant soluble human TNF receptor (II)-IgG1 fusion protein (shu-TNF-R). These data indicate regulation of IL-12 by an autocrine-dependent feedback loop, providing an additional mechanism whereby shu-TNF-R, now used in clinical trials in MS, may be exerting its effect. (*J. Clin. Invest.* 1996. 98: 1539–1543.) Key words: central nervous system • multiple sclerosis • tumor necrosis factor • polymerase chain reaction • flow cytometry

Introduction

Microglial cells are a resident cell population within the central nervous system (CNS). During the course of inflammatory diseases of the CNS, including multiple sclerosis (MS)[1] and HIV-encephalopathy, the cells undergo a morphologic change into an activated phenotype (1). In this state, these cells are demonstrated to express an array of cell surface molecules and cytokines which can participate in regulating immune reactivity or effecting tissue injury within the CNS (2).

The cytokine IL-12 can be detected in active lesions in cases of MS (3). This cytokine, a heterodimeric 70-kD protein consisting of a 35- and a 40-kD subunit is known to be produced by activated macrophages and B cells (4). IL-12 promotes the acquisition of a Th1- cytokine secreting profile by CD4$^+$ T cells (5–7). Such cells secrete IFN-γ, TNF-α/β, and IL-2 and are associated with a cell-mediated immune response. In contrast, Th2 cells secrete the regulatory cytokines IL-4 and IL-10 and promote humoral immune responses. Myelin reactive T cells of the Th1 phenotype are the required cells for adoptive transfer of disease in experimental autoimmune encephalomyelitis (EAE) (8, 9). Simultaneous administration of myelin reactive Th2 cells inhibits disease development (8). CD4$^+$ T cells, recovered from the CNS of animals in the acute phase of EAE induced by immunization with myelin antigens, show predominantly a Th1 cytokine profile (8, 10). Whether only Th1 primed cells infiltrate the CNS or whether brain resident cells such as the microglia induce such a transition is a matter of speculation.

We have shown that adult human microglial cells undergo morphologic changes, indicative of an activated phenotype closely resembling that seen in the inflamed CNS, when kept in dissociated cell culture. Furthermore, these cells upregulate expression of various surface molecules, such as CD14, HLA-DR, and B7-2, involved in immune regulation (2). In this study, we determined whether microglia were the human glial cells which could be induced to secrete IL-12 and the cytokine mechanisms regulating such production. We specifically focused on the role of TNF-α, a cytokine detected in acute lesions in EAE and MS, as an autocrine regulator of IL-12. Microglia are shown to express both p55 and p75 TNF receptors (11). We report that by blocking the action of autocrine TNF-α, with shu-TNF-R, we can inhibit LPS-induced production of IL-12 by human adult microglia.

Methods

Reagents. IFN-γ (100 U/ml) and TNF-α (100 U/ml) were obtained from Genzyme (Cambridge, MA). LPS (10 μg/ml) was from Sigma (Mississauga, ON). shu-TNF-R (6.3×10^5 U/ml) was a generous gift from A. Troutt (Immunex Corp., Seattle, WA). IL-12 and TNF-α ELISA kits were obtained from R&D Systems (Minneapolis, MN) and Immunocorp (Montréal, QC), respectively. Preliminary studies established that the above concentrations were optimal for our studies.

Source and preparation of human adult glial cells. Primary adult human glial cells were obtained from surgical resections performed for treatment of non–tumor-related intractable epilepsy. Tissues were

Address correspondence to J.P. Antel, M.D., Montréal Neurological Institute, 3801 University, Montréal, QC H3A 2B4 Canada. Phone: 514-398-8531; FAX: 514-398-7371; E-mail: MDAN@musica.mcgill.ca
Received for publication 10 May 1996 and accepted in revised form 31 July 1996.

1. *Abbreviations used in this paper:* EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/96/10/1539/05 $2.00
Volume 98, Number 7, October 1996, 1539–1543

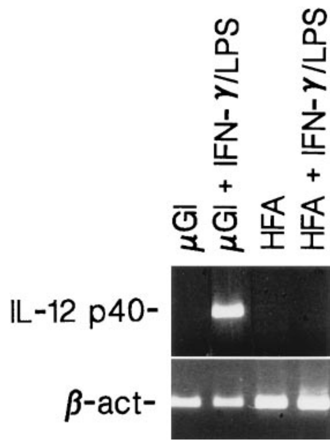

Figure 1. IL-12 p40 mRNA expression by activated microglial cells. Microglial and astroglial cells were analyzed under basal culture conditions or after stimulation for 24 h with LPS/IFN-γ. RNA was extracted for semiquantitative PCR analysis. The products were resolved on a 1.5% agarose gel and visualized by ethidium bromide. PCR for α-actin is used as an internal control. IL-12 p40 mRNA was not detected in nonstimulated microglia (μGl), but was detected in IFN-γ/LPS–treated microglia (*μGl + IFN-γ/LPS*). IL-12 p40 mRNA could not be detected in fetal human astroglia under either basal (*HFA*) or activated (*HFA + IFN-γ/LPS*) culture conditions. Data shown are from one of three representative experiments.

from regions requiring resection to reach the precise epileptic focus and were distant from the main electrically active site. Our method for isolating and culturing adult human microglial cells for use in dissociated cell culture has been described previously (12). Briefly, 1–3-mm^3 tissues were treated with 0.025% collagenase A and DNase (50 mg/ml) (both obtained from Boehringer Mannheim, Laval, QC) for 45 min, followed by mechanical dissociation by passage through a 130-μm nylon mesh. Cells were further separated on a linear 30% Percoll gradient (Pharmacia, Montréal, QC) (15,000 rpm for 30 min). The cells recovered from the interface contained a mixed glial cell population consisting of ~ 65% oligodendroglia, 30% microglia, and 5% astroglia. To enrich for microglia, the mixed cell population was suspended in Eagle's MEM, supplemented with 5% FCS, 2.5 U/ml penicillin, 2.5 mg/ml streptomycin, 2 mM glutamine, and 0.1% glucose (all from GIBCO, Burlington, ON) and left overnight in 25-cm^2 tissue culture flasks or 48-well plates (FALCON, Fisher Scientific, Montréal, QC) in a humid atmosphere at 37°C with 5% CO_2. The less adherent oligodendroglia were removed by gentle shaking. Remaining adherent cells, consisting of astroglia and microglia, were allowed to develop morphologically for 3 d. At this time, the less adherent astroglia were then floated off by rotary shaking for 5 h at 150 rpm. Remaining microglia (1.2×10^5 cells/cm^2) are of 95% purity, as assessed by immunocytochemistry (12).

Source and preparation of fetal human glial cells. These studies used 12–14-wk-old fetal specimens obtained following Medical Research Council of Canada approved guidelines. The central CNS tissues were stripped of meninges and blood vessels, mechanically dissociated with scalpel blades, and then treated with trypsin (0.25%) and DNase (50 mg/ml) at 37°C for 45 min. Dissociated tissue was passed through a 130-μm mesh, washed twice in PBS, and cells, initially consisting of astroglia, neurons, and sparse microglia, were plated directly onto poly-L-lysine–coated 35-cm^2 plastic culture dishes in MEM (see above). Cultures were split when confluent. These cultures were found to be highly enriched for astroglia, the main proliferating cells in these cultures, as determined by glial fibrillary acidic protein immunoreactivity.

Isolation of peripheral blood–derived cells. PBMC were isolated from healthy adult volunteer donors by density gradient centrifugation using Ficoll-Hypaque (Pharmacia). The cells were then washed twice with PBS and cultured for 1 h in RPMI 1640 medium (GIBCO) supplemented with 10% FCS, 2.5 mg/ml penicillin, 2.5 mg/ml streptomycin, and 2 mM glutamine in 75-cm^2 tissue culture flasks (FALCON, Fisher). The nonadherent cells were removed by gentle shaking. The adherent monocytes were of 95% purity.

Semiquantitative PCR analysis. Total RNA was isolated using TRIZOL reagent (GIBCO). To transcribe into cDNA, 3 μg RNA, 3.3 mM random hexamer primers (Boehringer, Mannheim, Germany), RT buffer, 3 mM dNTPs, 400 U MMLV-reverse transcriptase (all from GIBCO), 0.6 μl RNA guard, and 3 mM DTT (both from Pharmacia) were added to a total volume of 32 μl. The reaction mixture was incubated for 1 h at 42°C followed by a 10-min incubation at 75°C. Primers used for PCR reaction were obtained from the Sheldon Biotech Center (Montréal, QC) and had the following sequences: IL-12 p40 forward 5′-GCCATGTGTCACCAGCAGTT-3′, reverse 5′-CCAGCAGGTGACGTCCAG-3′, and β-actin forward 5′-ATGCCATCCTGCGTCTGGACCTGGC-3′, reverse 5′-AGCATTTGCGGTGCACGATGGAGGG-3′. The primers for IL-12 p40 and β-actin were constructed to generate fragments of 363 and 606 bp, respectively. 200 ng cDNA was added to the reaction mixture containing PCR buffer, 0.5 mM dNTPs (GIBCO), 50 pmol of either primer set for IL-12 p40 or β-actin, and 0.5 μl *Taq* polymerase (GIBCO). The reaction mixture was completed with H_2O to a total volume of 50 μl and was then overlaid with 100 μl of heavy mineral oil. Samples were placed in a thermocycler (Perkin Elmer Cetus Corp., Norwalk, CT) for 28 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min. Following amplification, 20 μl of each sample was electrophoresed on a 1.5% agarose gel (GIBCO). The bands were visualized with ethidium bromide. For quantification purposes 2.5 μCi of [^{32}P]dCTP (NEN Du Pont, Mississauga, ON) was added to the reaction mixture prior to PCR and the bands were analyzed using phosphorimaging and Image Quant software (Molecular Dynamics, Sunnyvale, CA). Results were presented as x-fold increase or decrease = $\Delta^{treated}/\Delta^{ctrl}$ (Δ = Phosphorimager units [PIU] IL-12 or TNF-α/PIU β-actin $\times$ 100).

Results

IL-12 production by microglia as a function of activation state. For these studies human adult microglia were allowed to develop morphologically in culture for 3 d after isolation. Fig. 1

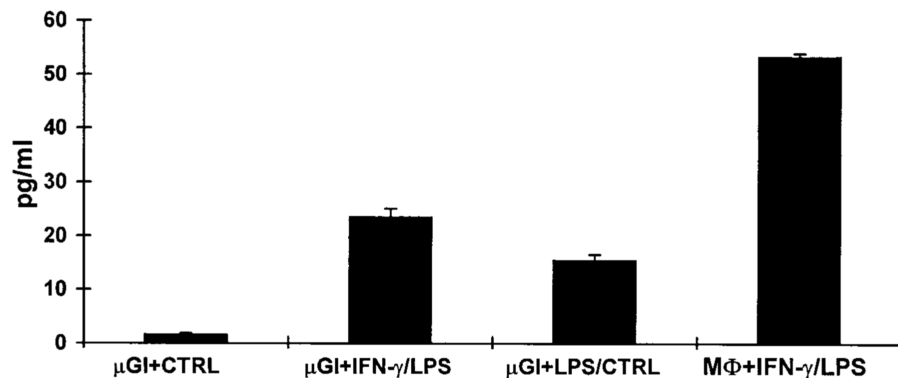

Figure 2. IL-12 p70 production by activated microglia. Microglia cells (*μGl*) were analyzed under basal culture conditions or after stimulation for 24 h either with LPS and IFN-γ or LPS and carrier buffer. The supernatants were harvested from cultures containing 1.2×10^5 cells/cm^2. Production of IL-12 p70 was assessed by ELISA. Supernatant derived from IFN-γ/LPS–stimulated monocytes (*MΦ*) was used as a positive control. 7.8 pg/ml was the lowest level of detectability. Results are expressed as ±SEM of duplicate experiments.

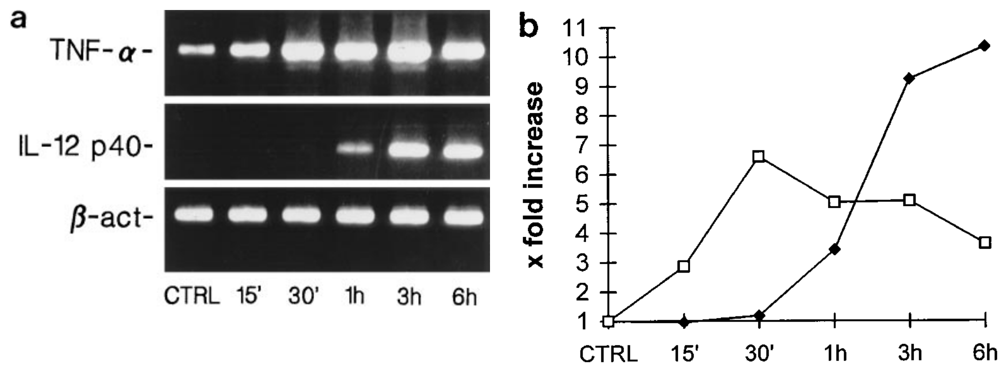

Figure 3. Kinetics of IL-12 p40 and TNF-α mRNA expression by LPS-activated microglia. Microglial cells were treated with 10 μg/ml LPS and the RNA was extracted at times ranging from 15 min to 6 h. Control cultures (*CTRL*) are nonstimulated microglia. RNA was extracted for semiquantitative PCR analysis. PCR for β-actin is used as an internal control. The PCR products were resolved on a 1.5% agarose gel and visualized by ethidium bromide (*a*). *b* shows quantification of the PCR products by Phosphorimager scanning. For quantification purposes, [^{32}P]dCTP was added to the PCR reaction (TNF-α, □; IL-12 p40, ◆). Data shown are from one of three representative experiments.

indicates that microglia maintained under basal culture conditions do not express detectable levels of IL-12 p40 mRNA. After 24 h of activation with IFN-γ/LPS, expression can be readily detected. Subsequent studies (see Fig. 5) indicated that LPS alone induces expression, although to a lesser extent. Incubation with IFN-γ alone was insufficient to induce such a response. We could not detect IL-12 p40 mRNA in human fetal astroglia, even when stimulated with IFN-γ/LPS, either at 24 h or at earlier time points (6 and 12 h; data not shown).

Secretion of IL-12 p70 heterodimer by microglia was assessed by ELISA (Fig. 2). IL-12 p70 could not be detected in microglial cultures maintained under basal conditions. Supernatants of microglial cultures (1.2×10^5 cells/cm^2) stimulated for 24 h with IFN-γ/LPS contain a mean 24 pg/ml of IL-12. Microglia stimulated with LPS alone produced a mean of 16 pg/ml. Activated monocytes, used as a positive control, were found to express higher levels of IL-12 p70 than did the microglia. These results show that microglia are the resident cells within the CNS with the ability to produce IL-12.

Kinetics of TNF-α and IL-12 expression by human adult microglia. To establish that TNF-α is produced prior to IL-12 by microglia, we used semiquantitative PCR analysis and visualization with ethidium bromide. TNF-α mRNA was undetectable in immediately ex vivo microglia (data not shown). However, as shown in Fig. 3 *a*, microglia maintained under basal culture conditions express low levels of TNF-α mRNA. After incubation with 10 μg/ml LPS, the cells rapidly upregulate TNF-α production (15 min). In contrast to TNF-α mRNA, IL-12 mRNA was first detected 1 h after stimulation with LPS. IL-12 message gradually increases over the 6-h time period studied. Fig. 3 *b* shows quantification of the PCR products as described in Methods.

To quantify the increased TNF-α and IL-12 protein production by activated microglia, microglial cultured in 48-well plates were treated with LPS and the supernatant was harvested at different time points after treatment. The results are expressed as x-fold increase compared with values derived from microglia cultured in the presence of carrier buffer. As shown in Fig. 4, a 12-fold increase of TNF-α can be first detected by ELISA in the supernatants of LPS-activated microglia, 3 h after stimulation. Detectability of TNF-α protein production peaks between 12 and 18 h. IL-12 p70 first becomes detectable after 12 h. 18 h after LPS treatment there is a 21-fold increase. These data show that TNF-α is expressed prior to IL-12.

Regulation of IL-12 production by endogenous TNF-α. To determine whether autocrine-derived TNF-α is necessary to induce IL-12 production as a response to the bacterial endotoxin LPS, we added 6.3×10^5 U/ml of shu-TNF-R to the cultures simultaneously with the addition of LPS. Human IgGl and carrier buffer was used as a control. After 24 h, cells were collected for RNA extraction and the supernatant was harvested for the protein assay. In cultures containing LPS and shu-TNF-receptor, IL-12 p40 mRNA levels were decreased compared with control cultures (Fig. 5). Addition of TNF-α (100 U/ml) without LPS to the cultures did not induce IL-12 production. A 3-fold increase of IL-12 p70 could be detected in shu-TNF-receptor–treated cultures compared with a 10-fold increase by microglia treated with LPS alone (LPS/CTRL) (Fig. 6). Results could be reproduced using a polyclonal anti–

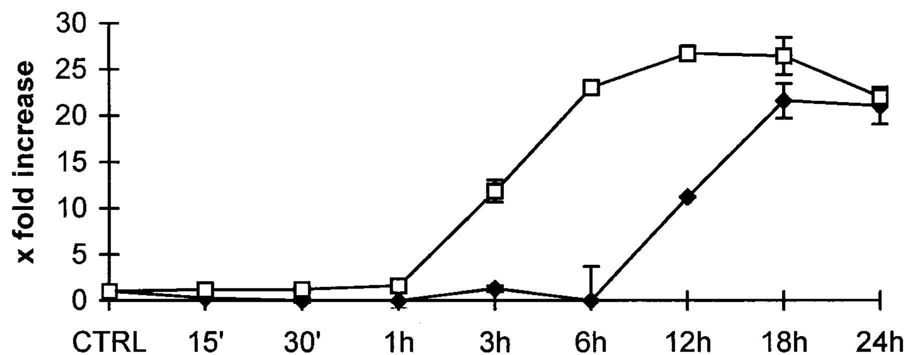

Figure 4. Kinetics of IL-12 and TNF-α protein production by LPS-activated microglial cells. Microglial cells were treated with 10 μg/ml LPS and the culture medium was harvested at times ranging from 15 min to 24 h. Control cultures (*CTRL*) are nonstimulated microglia. Protein concentrations were analyzed by specific ELISA. Kinetics of TNF-α (□) and IL-12 p70 (◆) production is presented as fold increase (±SEM) compared with control values of duplicate experiments.

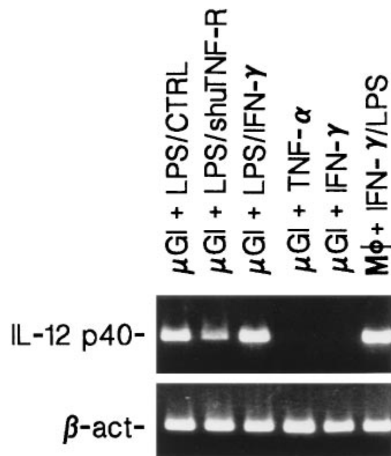

Figure 5. Effect of shu-TNF-R on IL-12 p40 mRNA expression by LPS-activated microglial cells. Microglia (*μGl*) were treated with either LPS, LPS/IFN-γ, TNF-α, or IFN-γ for 24 h. LPS-stimulated microglia were incubated with either IgG1/carrier buffer (*CTRL*) or shu-TNF-R (6.3 × 10^5 U/ml). Monocytes (*M*ϕ) activated with IFN-γ/LPS were used as a positive control. RNA from the cells were extracted for semiquantitative PCR analysis of IL-12 p40. The products were resolved on a 1.5% agarose gel and visualized by ethidium bromide. PCR for β-actin was used as an internal control. Data shown are from one of three representative experiments.

TNF-α rabbit serum (data not shown). Depletion of TNF-α in the microglial cultures by use of shu-TNF-R did not have any impact on morphology or viability within 2 wk of observation. Addition of shu-TNF-R to LPS/IFN-γ activated microglia did not alter IL-12 expression (data not shown).

Discussion

The results of our in vitro studies of microglia and astroglia suggest that microglia are the apparent endogenous cell source of IL-12 in the CNS. Since human adult astroglia have not yet been successfully purified, we used fetal astroglia as a replacement for adult astroglia in our studies. Human fetal astroglia have been shown to produce an array of cytokines. In comparative studies fetal astroglia have been indicated as a more potent source of proinflammatory cytokines than adult astroglia (13). We could not detect IL-12 in fetal human stroglia even after activation with IFN-γ/LPS. IL-12 production by microglia is dependent on the activation state of the cell. We could not detect IL-12 mRNA in microglia under basal culture conditions even though these cells show some degree of activation compared with immediately ex vivo microglia as determined by upregulation of MHC class II, B7-2, and CD14 molecules and by TNF-α mRNA production (2). We could detect IL-12 mRNA and protein following LPS stimulation. IFN-γ, a cytokine released by T cells, augments LPS-induced IL-12 production but does not induce detectable levels of IL-12 mRNA. IFN-γ can induce upregulation of other molecules, such as MHC class II and B7-1. Differential regulation of immune regulatory molecules expressed by microglia could result in altered functional properties of these cells in shaping the immune response that occurs within the CNS. The synergistic effect between IFN-γ and LPS on IL-12 secretion exemplifies how molecules likely to be present within the inflamed or infected CNS can cooperate in modulating microglial function.

Our findings indicate that microglia-derived TNF-α acts as an autocrine regulator of IL-12 production. TNF-α expression by LPS-treated microglia precedes IL-12 expression. Depleting TNF-α from activated microglial cultures by use of shu-TNF-R inhibits production of IL-12 p70 protein. The observation that TNF-α alone does not induce IL-12 production suggests that TNF-α is acting in concert with an additional event, triggered by LPS. Addition of shu-TNF-R to the LPS/IFN-γ activated microglia does not inhibit IL-12 production, indicating that INF-γ can bypass the TNF-dependent, LPS-induced regulatory circuit. Endogenous TNF-α does not increase IL-12 production by LPS/IFN-γ activated microglia. These data are in accordance with studies on murine bone marrow–derived macrophages (14). Macrophages needed to be primed with either IFN-γ or TNF-α to produce IL-12 as a response to LPS or *Mycobacterium bovis* infection. Macrophages derived from IFN-γ as well as TNF-1 receptor knock-out mice were not able to respond to a mycobacterial infection with IL-12 production. These findings suggest that IL-12 expression by macrophages is controlled by at least two different inducers: the endotoxins from the bacteria themselves and the inflammatory cytokines TNF-α or IFN-γ. These multiple control mechanisms are likely to be beneficial to the host, in that accidental triggering of IL-12 production via one of these pathways alone could result in a cytotoxic response and tissue damage.

Production of IL-12 represents a further mechanism whereby microglia can function as regulators of immune activity within the CNS. Previous studies indicate that activated microglia express the crucial surface molecules required for their function as competent antigen presenting cells for T cells which enter the CNS (2). The constitutive expression of B7-2 but not B7-1 on microglia under noninflammatory conditions would repress

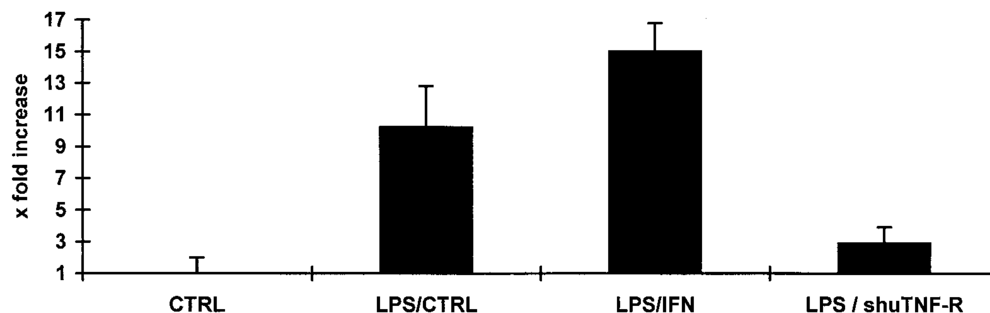

Figure 6. Effect of shu-TNF-R on IL-12 p70 production by LPS-activated microglial cells. Microglial cells were cultured either with IgG1/carrier buffer (*CTRL*) or stimulated for 24 h with either LPS and IgG1/carrier buffer (*LPS/CTRL*), LPS and IFN-γ or LPS and shu-TNF-R. The supernatants were harvested from cultures containing 1.2 × 10^5 cells/ml. Results are presented as x fold increase (±SEM) compared with control values of duplicate experiments.

a Th1 response within the CNS. Upregulation of B7-1 on microglia treated in vitro with LPS/IFN-γ, and in situ under inflammatory conditions, would favor a Th1 response. IL-12 production by microglia may be an even more potent factor which modulates the cytokine repertoire of infiltrating T cells. The identification of a cytokine network which regulates the production of IL-12 by microglia provides a potential means to manipulate its production. Administration of anti–IL-12 antibodies has been used to block development of EAE (15). Soluble TNF receptor is in use currently in clinical trials as a therapy for MS. The TNF-dependent IL-12 regulatory network described in this report represents a potential target for its action.

Acknowledgments

This work was supported by a grant from the Multiple Sclerosis Society of Canada.

References

1. Suzumura, A., T. Marunouchi, and H. Yamamoto. 1991. Morphological transformation of microglia in vitro. *Brain Res.* 545:301–306.

2. Becher, B., and J.P. Antel. 1996. Comparison of functional and phenotypic properties of immediately *ex vivo* and cultured human adult microglia. *GLIA*. In press.

3. Windhagen, A., J. Newcombe, F. Dangond, C. Strand, M.N. Woodroofe, M.L. Cuzner, and D.A. Hafler. 1995. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J. Exp. Med.* 182:1985–1996.

4. Trinchieri, G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* 13:251–276.

5. Hsieh, C.H., S.E. Macatonio, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of TH1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophage. *Science* (*Wash. DC*). 260:547–549.

6. Brunda, M.J. 1994. Interleukin-12. *J. Leukocyte. Biol.* 55:280–288.

7. Scott, P. 1993. IL-12: initiation cytokine for cell-mediated immunity. *Science* (*Wash. DC*). 260:496–497.

8. Cua, D.J., D.R. Hinton, and S.A. Stohlman. 1995. Self-antigen-induced Th2 responses in experimental allergic encephalomyelitis (EAE)-resistant mice. Th2-mediated suppression of autoimmune disease. *J. Immunol.* 155:4052–4059.

9. Kuchroo, V.K., M.P. Das, J.A. Brown, A.M. Ranger, S.S. Zamvil, R.A. Sobel, H.L. Weiner, N. Nabavi, and L.H. Glimcher. 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell.* 80:707–718.

10. Renno, T., M. Drakowski, C. Piccirillo, J.Y. Lin, and T. Owens. 1995. TNF-alpha expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis. Regulation by Th1 cytokines. *J. Immunol.* 154:944–953.

11. Wilt, S.G., E. Milward, J.M. Zhou, K. Nagasato, H. Patton, R. Rusten, D.E. Griffin, M. O'Connor, and M. Dubois-Dalcq. 1995. In vitro evidence for a dual role of tumor necrosis factor-alpha in human immunodeficiency virus type 1 encephalopathy. *Ann. Neurol.* 37:381–394.

12. Yong, V.W., and J.P. Antel. 1992. Culture of cells from human brain biopsies. *In* Protocols for Neural Cell Culture. S. Fedoroff and A. Richardson, editors. Plenum Publishing Corp., New York. 35–42.

13. Lafortune, L., J. Nalbantaglu, and J.P. Antel. 1996. Expression of TNF-a and IL-6 mRNA in adult human astrocytes: comparison with adult microglia and fetal astroglia. *J. Neuropathol. Exp. Neurol.* 55:515–521.

14. Flesch, I.E., J.H. Hess, S. Huang, M. Aguet, J. Rothe, H. Bluethmann, and S.H. Kaufmann. 1995. Early interkeukin 12 production by macrophages in response to mycobacterial infection depends on interferon gamma and tumor necrosis factor alpha. *J. Exp. Med.* 181:1615–1621.

15. Leonard, J.P., K.E. Waldburger, and S.J. Goldman. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J. Exp. Med.* 181:381–386.