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Microglia Are More Efficient Than Astrocytes in Antigen Processing and in Th1 But Not Th2 Cell Activation

Francesca Aloisi, Francesco Ria, Giuseppe Penna, and Luciano Adorini

Microglia and astrocytes, two glial cell populations of the central nervous system, present Ag and stimulate T cell proliferation, but it is unclear whether they preferentially activate Th1 or Th2 responses. We have investigated the efficiency of microglia and astrocytes in the presentation of OVA peptide 323-339 or native OVA to Th1 and Th2 cell lines from DO11.10 TCR transgenic mice. Upon stimulation with IFN-γ, microglia express MHC class II molecules, CD40, and ICAM-1 and efficiently present OVA 323-339, leading to T cell proliferation and production of IL-2 and IFN-γ by Th1 and of IL-4 by Th2 cells. IFN-γ-treated astrocytes, which express MHC class II and ICAM-1, present OVA 323-339 less efficiently to Th1 cells but are as efficient as microglia in inducing IL-4 secretion by Th2 cells. However, astrocytes are much less potent than microglia in presenting naturally processed OVA peptide to either T cell subset, indicating inefficient Ag processing. The capacity of astrocytes and microglia to stimulate Th1 and Th2 cells depends on their MHC class II expression and does not involve ICAM-1, B7-1, or B7-2 molecules. However, CD40-CD40L interactions contribute to Th1 activation by microglia. These data suggest that microglia may play a role in the activation of Th1 and Th2 cells, whereas astrocytes would restimulate mainly Th2 responses in the presence of appropriate peptides. This differential capacity of brain APC to restimulate Th1 and Th2 responses may contribute to the reactivation and regulation of local inflammatory processes during infectious and autoimmune diseases.


The presence of an intact blood brain barrier that limits the passage of T cells into the central nervous system (CNS), and the low expression of MHC class II and T cell adhesion/costimulatory molecules in normal conditions render the CNS an unsuitable site for T cell priming (1, 2). However, T cells activated in peripheral lymphoid organs can cross the blood brain barrier and recognize target Ags within the CNS (3, 4). In this scenario, resident CNS APC are likely to play a major role in T cell reactivation and may contribute to the propagation and maintenance of immune responses in multiple sclerosis (MS), a chronic inflammatory disease of the CNS, or during certain viral infections (5, 6).

T lymphocytes have been subdivided into two distinct subpopulations that differ in their cytokine profile and effector functions. Th1 lymphocytes, characterized by IL-2, IFN-γ, and TNF-β secretion, induce cell-mediated immune responses, whereas Th2 lymphocytes selectively produce IL-4, IL-5, IL-10, and IL-13 and promote humoral immune responses (7, 8). Th1-dominated responses are often associated with inflammation and tissue destruction that lead to organ-specific autoimmune diseases (5, 9, 10). Autoreactive CD4+ T lymphocytes exhibiting a Th1 phenotype are thought to play a role in the pathogenesis of MS (11) and experimental allergic encephalomyelitis, an animal model of CNS autoimmune disease (12, 13). Conversely, several cytokines produced by Th2 lymphocytes, such as IL-4, IL-10, and IL-13, have anti-inflammatory activities. Activation of Th2 lymphocytes may inhibit CNS inflammation and limit the noxious effects of Th1-mediated immunity, as observed in experimental allergic encephalomyelitis (14, 15). IL-12 promotes the differentiation of naïve T cells to Th1 and is a potent costimulus for the activation of differentiated Th1 cells (16, 17), whereas IL-4 drives the development of Th2 responses. In addition, the development of Th1 and Th2 lymphocytes from a common undifferentiated precursor depends on several other factors, including the avidity of TCR-ligand interaction and costimulatory signals delivered by the APC (7).

Within the CNS parenchyma, microglia, a type of highly differentiated and quiescent tissue macrophage, constitutively express MHC II molecules (18–20). MHC class II expression is greatly increased on reactive microglia in response to neuronal damage, infection, or inflammation (21, 22). IFN-γ and/or LPS stimulate cultured microglia to express MHC class II as well as adhesion/costimulatory molecules and to produce bioactive IL-12 (23–27). In vitro, activated microglia are able to prime alloreactive T cell responses (24, 28) and to stimulate T cell lines to proliferate and secrete cytokines (23, 29, 30). Adhesion/costimulatory molecules, such as ICAM-1, CD40, CD80, and CD86, are also expressed by reactive microglia in inflammatory MS lesions (25, 31–33), suggesting a role for these APC in T cell activation in vivo.

Astrocytes, the major CNS glial cell type, have also been proposed to have a role as APC. While MHC class II expression on astrocytes in situ remains controversial (34–39), astrocytes stimulated in vitro by IFN-γ and TNF-α express MHC class I and II molecules as well as ICAM-1, vascular cell adhesion molecule-1, and LFA-3 (24, 40–43). Conflicting data have been reported on the capacity of cultured astrocytes to activate T cell responses. Depending on the source of astrocytes and the type of responding...
T cell, astrocytes have been shown to act as stimulators or inhibitors of the proliferation of primed T cells (29, 41, 43–46) and to be able or unable to stimulate the proliferation of naive T cells (24, 28, 46, 47). Conflicting data also exist on the type of T cell co-stimulatory signals (e.g., B7 and IL-12) expressed or inducible in astrocytes (25–27, 47–49).

T cell activation within the CNS is still poorly understood and no information is yet available on the efficiency of CNS APC to restimulate Th1 and Th2 responses. In this study, we have determined the capacity of microglia and astrocytes from BALB/c mouse forebrains to process and present Ag to OVA-specific TCR transgenic Th1 and Th2 cells, leading to T cell proliferation and cytokine secretion.

Materials and Methods

Mice

BALB/c mice were purchased from Charles River (Calco, Italy). One-day-old mice were used for the preparation of brain cell cultures, whereas 2- to 3-mo-old female mice were used for the preparation of spleen cells. DO11.10 TCR transgenic mice on BALB/c background (50) were kindly provided by Dr. D. Y. Loh (Hoffmann-La Roche, Nutley, NJ). In these transgenic mice, 95 to 100% of the CD4+ T cells are Vβ8.1,2+ (51) and express a TCR-β specific for OVA peptide 323–339 bound to I-A\(^d\).

Cell cultures

Primary mixed glial cultures were established from the forebrains of 1-day-old BALB/c mice, following previously published procedures (48). Forebrains were carefully freed of meninges, chopped into 0.25-mm sections, and dissociated by a mild trypsinization procedure and gentle mechanical disruption with a Pasteur pipette. The cells were seeded into poly-l-lysine (10 \(\mu\)g/ml)-coated 175-cm\(^2\) flasks at the density of 4 \(\times 10^5\) cells/cm\(^2\) and grown at 37°C in a 92% air-8% CO\(_2\) humidified atmosphere in DMEM (HyClone, Cramlington, U.K.) containing 0.45-\(\mu\)m filtered, 10% FCS Myocyte (Life Technologies, Gaithersburg, MD), 2 \(\mu\)M glutamine (Bio-Whittaker, Verviers, Belgium), penicillin (100 U/ml), and streptomycin (100 \(\mu\)g/ml). The medium was replaced after 24 h and then every 3 days when preparing astrocyte cultures, or only once after 4 days when preparing microglial cultures. After about 10 days in vitro, microglial cells were detached from the astroglial monolayer by rapid (15–30 s) and gentle mechanical disruption with a Pasteur pipette. The cells were seeded into poly-l-lysine-coated plastic surfaces.

Spleen cells were prepared from normal BALB/c mice, irradiated with 3000 rad, and seeded in 96-well flat-bottom culture plates in RPMI 1640 (Life Technologies) supplemented with 50 \(\mu\)M 2-ME, 2 mM L-glutamine, 10 \(\mu\)g/ml anti-mouse IL-12 mAb (10F6; Hoffmann-La Roche) and 10 NG/ml anti-mouse IL-4 (51) and 10% FCS (Sigma, St. Louis, MO).

T cell lines

CD4+ cells were positively selected from inguinal and mesenteric lymph nodes of naive DO11.10 TCR transgenic mice by anti-CD4-coated magnetic microbeads (Miltenyi-Biotec GmbH, Bergish, Germany). CD4+ T cells (2 \(\times 10^5\) cells/well) were cultured with OVA peptide 323-339 (0.3 \(\mu\)M), synthesized as previously described (52), and mitomycin C-treated BALB/c splenocytes (5 \(\times 10^5\) cells/well) as APC in a total volume of 2 ml in 24-well plates, in the presence of either 0.1 ng/ml recombinant mouse IL-12 (Hoffmann-La Roche, Nutley, NJ) and 10 \(\mu\)g/ml anti-mouse IL-4 (1B11; American Type Culture Collection (ATCC), Rockville, MD), or 20 ng/ml mouse recombinant IL-4 (Hoffmann-La Roche AG, Basel, Switzerland) and 10 \(\mu\)g/ml anti-mouse IL-12 mAb (10F6; Hoffmann-La Roche) to obtain Th1 or Th2 cell lines, respectively. Cells were cultured in RPMI supplemented with 50 \(\mu\)M 2-ME, 2 mM L-glutamine, 50 \(\mu\)g/ml gentamicin, and 10% FCS (Sigma). After 3 days in vitro, T cells were expanded and grown in complete medium containing 10 ng/ml of recombinant human IL-2 (Hoffmann-La Roche AG).

Ag presentation

Varying numbers of microglia and astrocytes were seeded in 96-well flat-bottom tissue culture plates. After 16 h, the culture medium was replaced with fresh medium (DMEM/10% FCS) without or with cytokines or LPS, and cells were incubated at 37°C for additional 24 to 48 h. Mouse recombinant cytokines used in this study were: IFN-γ (sp. act. 1 \(\times 10^7\) U/mg), TNF-α (sp. act. 4 \(\times 10^5\)–4 \(\times 10^7\) U/mg), and IL-1β (sp. act. 4 \(\times 10^5\)–1 \(\times 10^7\) U/mg), all from Genzyme, Cambridge, MA. LPS (from Escherichia coli, serotype 026:B6) was purchased from Sigma. Immediately before the addition of T cells from transgenic mice, astrocytes and microglia were gently washed three times with DMEM/10% FCS to completely remove stimulatory agents. Spleen cells were labeled at densities ranging from 3 \(\times 10^6\) to 1 \(\times 10^7\) cells/well. TCR transgenic Th1 and Th2 cells were collected from 6- to 7-day-old cultures and added (5 \(\times 10^4\)) in RPMI/10% FCS (supplemented as described above) to wells containing the different APC in the presence of different doses of OVA 323-339 peptide (0.001–0.3 \(\mu\)M) (52) or native OVA (grade V, from Sigma) (0.01–30 \(\mu\)M). For inhibition of T-cell proliferation, the following mAbs (10 \(\mu\)g/ml) were added to the cultures 30 min before addition of T cells: B21.22, anti-I-A\(^d\) (ATCC); 14-4–4S, anti-I-E\(_{\alpha}\) (ATCC); YN1/17.4, anti-ICAM-1 (ATCC); 1G10, anti-B7-1 (PharMingen, San Diego, CA); 37.51, anti-CD28 (PharMingen); 2D10, anti-B7-2 (53); MR1, anti-CD40 ligand (PharMingen); hamster IgG (PharMingen); rat IgG2a (PharMingen); rat IgG2b (ATCC). For analysis of T cell-derived cytokines, supernatants from triplicate cultures were harvested after 24 or 48 h, centrifuged at 1200 rpm and stored at -20°C until used for cytokine determination. For proliferation assays, microglia, astrocytes, and spleen cells were irradiated (3000 R) immediately before addition of T cells and Ag. Proliferation was measured by incorporation of 0.5 to 1 \(\mu\)Ci/well (\[^{3}H\]thymidine (Amersham Italia, Milan, Italy; sp. act. 25 Ci/mmol)) during the last 16 h of a 48-h incubation period. Radioactivity was detected using a Packard Topcount microplate scintillation counter (Packard Instrument, Meriden, CT).

Cytokine assays

IL-2, IFN-γ, and IL-4 were quantified by a two-site sandwich ELISA as previously described (54). All mAbs, except anti-IFN-γ, were purchased from PharMingen. The anti-IFN-γ mAb used for capture was AN.18.17.24 (55) and the mAb used for detection was peroxidase-conjugated XMG1.2 (56). Cytokines were quantified from three to four titration points using standard curves generated by purified recombinant mouse cytokines (IFN-γ and IL-4 from Hoffmann-La Roche AG, and IL-2 from PharMingen) and results expressed as cytokine concentration in ng/ml. Detection limits for all cytokines were in the range of 7 to 15 pg/ml.

Intracellular staining for IFN-γ and IL-4 production

Polarized Th1 and Th2 cells were restimulated with PMA (50 ng/ml) and ionomycin (0.75 \(\mu\)g/ml) for 4 h at 37°C, with 10 \(\mu\)g/ml of brefeldin A (Sandoz, Basel, Switzerland) for the last 30 min. After fixation for 20 min at room temperature in 2% paraformaldehyde, the cells were stained for intracytoplasmic IFN-γ and IL-4 using the method described by Oksenhendler et al. (57). All incubations were performed at room temperature in PBS containing 5% FCS, 0.5% saponin (Sigma), and 0.1% sodium azide. After washing and 10 min of preincubation in PBS/FCS/saponin, cells were incubated with FITC rat anti-mouse IFN-γ (XM1G2.1, PharMingen) and phycoerythrin (PE) rat anti-mouse IL-4 (1B11; PharMingen) or with FITC- and PE-labeled rat IgG1 isotype controls (R3-34; PharMingen). After 30 min, cells were washed twice with PBS/FCS/saponin and then with PBS/5% FCS to allow membrane closure. Cell membranes were then stained with Cy-Chrome-labeled anti-CD4 (L3T4; Pharmingen) for 15 min. Analysis was performed with a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with CELLQUEST software. In all, 30,000 events were acquired.

Flow cytometry

Sixteen hours after isolation from primary cultures, microglia and astrocyte cultures (5 \(\times 10^6\) cells/35-mm diameter culture dishes) received fresh culture medium or medium containing cytokines or LPS, as above. After 24 to 48 h of incubation, cells were washed with DMEM, preincubated on ice for 30 min with DMEM containing 30% goat serum, and then incubated on ice for another 30 min with FITC- or PE-conjugated mAbs in DMEM/30% goat serum to inhibit binding to FcR. The following fluorochrome-conjugated mAbs (all from PharMingen) were used at 1:100 to 1:200 dilution:
then for an additional 30 min with FITC goat F(ab') IgG2a; PharMingen) or isotype control Ab (rat IgG2a; PharMingen) and ICAM-1 mAb (IgG2a; Serotec, Oxford, U.K.), rat anti-mouse B7-1 (1G10, and B7-1 stainings, cells were incubated for 30 min with rat anti-mouse ICAM-1 mAb (IgG2a; Serotec, Oxford, U.K.), rat anti-mouse IgG2a, and FITC mouse IgG3 (all from PharMingen). For CD54 (ICAM-1) IgG2a). The background fluorescence was evaluated by staining the cells with isotype control Abs: PE rat IgG2b, FITC rat IgG2a, FITC mouse IgG2a, and FITC mouse IgG3 (all from PharMingen). For CD54 (ICAM-1) and B7-1 stainings, cells were incubated for 30 min with rat anti-mouse ICAM-1 mAb (IgG2a; Serotec, Oxford, U.K.), rat anti-mouse B7-1 (1G10, IgG2a; PharMingen) or isotype control Ab (rat IgG2a; PharMingen) and then for an additional 30 min with FITC goat F(ab') IgG2a, anti-rat IgG (Organon Teknika-Cappell, Durham, NC). At the end of the incubations, cells were washed three times with DMEM. Microglial cells were detached from the culture dishes using a cell scraper, whereas astrocytes were detached by gentle pipetting after incubating at 4°C for 5 to 10 min in PBS/0.02% EDTA. Cells were centrifuged and resuspended in 0.3 ml PBS/0.5% paraformaldehyde. For detection of the astrocyte-specific intermediate filament protein glial fibrillary acidic protein (GFAP), microglia and astrocytes were detached from the culture dishes as above and fixed with 4% paraformaldehyde in PBS for 5 min at room temperature. Cells were then permeabilized with Triton (0.1% in PBS) for 5 min and incubated with mouse anti-GFAP mAb (IgG, 1:200) (BioGenex Laboratories, San Ramon, CA) or control isotype Ab followed by FITC goat F(ab') IgG (1:200). Cells were analyzed on a FACSscan flow cytometer, and 3000 to 5000 events were acquired.

**Statistical analysis**

To quantitate the differences between microglia and astrocytes in their ability to stimulate Th1 and Th2 cytokine secretion, data were analyzed by Friedman nonparametric ANOVA (58), considering the experiments as blocks and the “cell type” by “cell density” combinations as repeated measures within experiments. The main effect of “cell type” and “cell density” and their interaction were assessed by the orthogonal partitioning of the overall Friedman $x^2$.

**Results**

**Immunocytochemical characterization of microglia and astrocyte cultures**

As the main goal of this study was to evaluate the efficiency of microglia and astrocytes in the restimulation of TCR transgenic T cells, we ascertained that all experiments were conducted using microglia and astrocyte cultures from BALB/c mouse forebrains devoid of any cross-contamination. Culture purity was assessed by surface staining with anti-Mac-1/CD11b, a marker for macrophages/microglia (59), and by intracellular staining with a mAb specific for the astrocytic intermediate filament protein GFAP (60).

Flow cytometric analysis revealed that microglia and astrocyte cultures contained 92 to 95% Mac-1+ cells and 95 to 98% GFAP+ cells, respectively (Fig. 1). Mac-1+ cells and GFAP+ cells were virtually absent in astrocyte and microglia cultures, respectively. Examination of the cultures by the fluorescence microscope after triple staining with anti-Mac-1, anti-GFAP mAb, and Hoechst 33258 fluorochrome (to label cell nuclei) confirmed that less than 1% of the total population expressed GFAP or Mac-1 in microglia or astrocyte cultures, respectively (not shown).

**TCR transgenic Th1 and Th2 cell lines**

Th1 and Th2 cell lines were generated from mice transgenic for the DO11.10 TCR, which recognizes the OVA epitope 323-339 bound to I-A$^d$ molecules. CD4+ lymph node T cells from TCR transgenic mice were cultured with mitomycin C-treated spleen cells from normal BALB/c mice and OVA peptide 323-339 in the presence of IL-12 and anti-IL-4 or IL-4 and anti-IL-12 mAb to promote the development of Th1 or Th2 cells, respectively. As shown in Figure 2, the cell lines obtained exhibit a clear Th1 or Th2 profile, as detected by intracellular staining for IFN-γ and IL-4 production. In both cell lines, >80% of total cells express the transgenic TCR, as determined by staining with the clonotype-specific mAb KJ1-26 (not shown).

**Constitutive and inducible expression of MHC class II and adhesion/costimulatory molecules on microglia and astrocytes**

To establish optimal in vitro conditions that enable microglia and astrocytes to present Ag, cultures were treated for 24 to 48 h with various agents (IFN-γ, LPS, TNF-α, IL-1β) that induce expression of MHC class II and adhesion/costimulatory molecules such as CD54 (ICAM-1), CD40, CD80 (B7-1), or CD86 (B7-2). Expression of these molecules was evaluated by flow cytometric analysis, as shown in Figure 3.

In unstimulated cultures, ICAM-1 is expressed on most astrocytes and microglia, whereas only microglia show low expression of I-E, B7-2, and CD40. In both cell types, IFN-γ (100 U/ml) induces expression of MHC class II I-A and I-E molecules, and enhances ICAM-1 expression, but has no effect on B7-1 or B7-2. The anti-B7-1 and anti-B7-2 mAbs used in this study stain the immortalized microglial cell line BV-2 (not shown), as previously reported (48). IFN-γ also up-regulates CD40 expression on microglia. In astrocyte cultures, TNF-α (10, 50 ng/ml) increases ICAM-1 expression and potentiates the inducing effect of IFN-γ on MHC class II and ICAM-1. IL-1β (100 U/ml) also enhances ICAM-1 on astrocytes, but does not affect IFN-γ-induced MHC class II expression (not shown). None of the surface molecules examined is induced on microglia by TNF-α or IL-1β (not shown). As expected, astrocytes do not respond to LPS (0.1, 1 μg/ml) (not shown).
high numbers (3 $\times$ 10^5$), plateau or even reduced values of cytokines are sometimes observed. In the presence of IFN-$\gamma$/LPS-treated microglia, which express high levels of B7-2, CD40, and ICAM-1 but are MHC class II negative, T cell cytokine secretion is similar to or lower than that elicited by unstimulated microglia. Cytokine secretion also is not induced by LPS-treated microglia (not shown). IFN-$\gamma$/TNF-$\alpha$-treated microglia are as efficient as IFN-$\gamma$-treated microglia in inducing cytokine secretion by Th1 and Th2 cells, whereas TNF-$\alpha$-treated microglia do not induce T cell activation at levels higher than those of unstimulated microglia (not shown). When activated by microglia, Th1 cells do not secrete IL-4, and Th2 cells secrete no IFN-$\gamma$ and little or no IL-2 (Fig. 4), confirming the polarization of the TCR transgenic T cells used. No cytokine production has ever been detected in the absence of OVA peptide or by microglia and T cells cultured alone (not shown).

Untreated astrocytes stimulate little or no cytokine secretion from Th1 and Th2 cells in the presence of 0.3 $\mu$M OVA 323-339 (Fig. 4). Astrocytes pretreated with IFN-$\gamma$ presentation OVA peptide and stimulate secretion of IL-2 and IFN-$\gamma$ by Th1 cells as well as IL-4 by Th2 cells. IFN-$\gamma$/TNF-$\alpha$-treated astrocytes are as effective as IFN-$\gamma$-treated astrocytes in stimulating cytokine secretion by either T cell subset, even if the combined cytokine treatment induces higher expression of MHC class II and ICAM-1 molecules (see Fig. 3). The stimulating effect of cytokine-activated astrocytes is dose dependent, being maximal at $\approx 3 \times 10^4$ cells/well. As expected, TNF-$\alpha$-, IL-1$\beta$-, or LPS-treated astrocytes, which are MHC class II negative, are as effective as unstimulated astrocytes. Treatment of astrocytes with IFN-$\gamma$ plus LPS or with IFN-$\gamma$ plus IL-1$\beta$ induces T cell activation similar to that induced by IFN-$\gamma$ treatment (not shown). As already observed with microglia, Th1 and Th2 cells secrete no IL-4 and no IFN-$\gamma$ or very little IL-2, respectively, when activated by astrocytes. No cytokine secretion is observed in the absence of OVA peptide or of T cells (not shown).

When T cell activation by microglia and astrocytes is compared, it is evident that IFN-$\gamma$-treated microglia are more efficient than IFN-$\gamma$-treated astrocytes in restimulating Th1 cells. The differences between Th1 cytokine production induced by activated microglia and astrocytes are statistically significant at all APC numbers tested ($p < 0.001$). It is noteworthy that secretion of IL-2 and IFN-$\gamma$ induced by 1 $\times$ 10^5 astrocytes is similar to that induced by 3 $\times$ 10^5 microglia, indicating that microglia are about 10-fold more efficient than astrocytes in stimulating Th1 cytokine secretion. In contrast, the two APC restimulate Th2 cells with a similar efficiency, although in some experiments, at the lowest cell densities tested (<1 $\times$ 10^4/well), microglia induce slightly higher IL-4 secretion than astrocytes.

**Involvement of MHC class II and adhesion/costimulatory molecules in the restimulation of Th1 and Th2 cells by IFN-$\gamma$-activated microglia and astrocytes**

To assess the contribution of MHC class II and adhesion/costimulatory molecules in the restimulation of cytokine secretion by Th1 and Th2 cells, IFN-$\gamma$-stimulated microglia or astrocytes (3 $\times$ 10^5/well) were cultured with OVA 323-339 and T cells in the presence of specific neutralizing Abs (10 $\mu$g/ml) or the same amount of their corresponding isotype controls. As shown in Figure 5, anti-I-A^b,d, but not anti-I-E mAb, inhibits almost completely the capacity of IFN-$\gamma$-stimulated microglia and astrocytes to induce IFN-$\gamma$ and IL-4 production by I-A^b,d-restricted, TCR transgenic Th1 and Th2 cells, respectively. In contrast, neutralizing mAbs against B7-1, B7-2, and CD28, the counter-receptor for B7 molecules on T cells, have no effect on the

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**Microglia present more efficiently than astrocytes the OVA peptide 323-339 to Th1 but not Th2 cells**

We next tested microglia and astrocytes for their ability to present OVA 323-339 and to induce cytokine secretion by Th1 and Th2 cells, either constitutively or after appropriate stimulation, as determined above. Polarized CD4$^+$ TCR transgenic cells were cultured with microglia or astrocytes in the presence of OVA 323-339. After 24 h, IL-2, IFN-$\gamma$, and IL-4 were quantified in culture supernatants by specific ELISA. In preliminary experiments, optimal T cell cytokine secretion was induced by 0.3 $\mu$M OVA 323-339 (not shown), and this dose was used in the following experiments.

As shown in Figure 4, in the presence of 0.3 $\mu$M OVA 323-339, high numbers (3 $\times$ 10^4 cells/well) of untreated microglia induce production of IL-2 and IFN-$\gamma$ by Th1 cells but little or no IL-4 by Th2 cells. Although I-A^d molecules are undetectable on unstimulated microglia by flow cytometry (see Fig. 3), in high cell density cultures low amounts of I-A^d or other adhesion/costimulatory molecules could be sufficient to drive Th1 activation. During the 24-h coculture period, microglial I-A^d expression could also be stimulated by IFN-$\gamma$, which is produced by Th1 but not Th2 cells. IFN-$\gamma$-treated microglia, which are MHC class II$^+$, CD40$^+$, and strongly ICAM-1$^+$, are much more potent than unstimulated microglia in presenting OVA 323-339 and efficiently activate both T cell subsets. At any cell density tested, IFN-$\gamma$-treated microglia induce the secretion of large amounts of IL-2 and IFN-$\gamma$ by Th1 cells and of IL-4 by Th2 cells. Stimulation of Th1 and Th2 cytokine production is dose dependent, maximal stimulation being obtained with $\approx 3 \times 10^4$ cells/well. However, at the highest cell density (1 $\times$ 10^5), plateau or even reduced values of cytokines are sometimes observed. In the presence of IFN-$\gamma$/LPS-treated microglia, which express high levels of B7-2, CD40, and ICAM-1 but are MHC class II negative, T cell cytokine secretion is similar to or lower than that elicited by unstimulated microglia. Cytokine secretion also is not induced by LPS-treated microglia (not shown). IFN-$\gamma$/TNF-$\alpha$-treated microglia are as efficient as IFN-$\gamma$-treated microglia in inducing cytokine secretion by Th1 and Th2 cells, whereas TNF-$\alpha$-treated microglia do not induce T cell activation at levels higher than those of unstimulated microglia (not shown). When activated by microglia, Th1 cells do not secrete IL-4, and Th2 cells secrete no IFN-$\gamma$ and little or no IL-2 (Fig. 4), confirming the polarization of the TCR transgenic T cells used. No cytokine production has ever been detected in the absence of OVA peptide or by microglia and T cells cultured alone (not shown).
capacity of microglia and astrocytes to induce cytokine secretion from either T cell subset. Both B7-1 and B7-2 mAbs inhibit the proliferation of TCR transgenic naive T cells stimulated by BALB/c dendritic cells (F. Ria and F. Aloisi, unpublished observations). These results are consistent with the lack of expression of B7-1 and B7-2 molecules on IFN-γ-activated BALB/c mouse microglia and astrocytes, as detected by flow cytometry (Fig. 3). Despite high expression of ICAM-1 on both microglia and astrocytes, anti-ICAM-1 mAb does not affect Th1 and Th2 cytokine secretion induced by either cell type. A modest (about 35%), but statistically significant reduction of IFN-γ secretion by Th1 cells, but not of IL-4 by Th2 cells, is observed when Th1 cells are activated by microglia in the presence of a mAb against CD40L, the counterreceptor for CD40 on T cells.

**FIGURE 3.** Constitutive and inducible expression of MHC class II and adhesion/costimulatory molecules on BALB/c microglia and astrocytes. Microglia were cultured for 24 h in the absence or presence of IFN-γ (100 U/ml), LPS (100 ng/ml), or IFN-γ plus LPS. Astrocytes were cultured for 48 h in the absence or presence of IFN-γ (100 U/ml), TNF-α (10 ng/ml), or IFN-γ plus TNF-α. Cells were stained for surface expression of the indicated molecules and examined by cytofluorometric analysis, as described in Materials and Methods. Stainings with control isotype mAbs are also shown (hatched histograms). The data shown are from representative experiments of two to three performed.
This is consistent with the expression of CD40 on IFN-γ-stimulated microglia (Fig. 3). Anti-CD40L mAb has no effect on Th1 or Th2 activation induced by IFN-γ-treated astrocytes. The above results indicate that the presence of MHC class II on CNS APC is critical for the stimulation of differentiated Th1 and Th2 cells, whereas CD40-CD40L interactions may play a role in the restimulation of Th1 cells by microglia.

Microglia are much more efficient than astrocytes in processing native OVA

Having established that microglia and astrocytes are able to present OVA 323–339 to Th1 and Th2 cells and that IFN-γ is the best stimulus for these APC to induce cytokine secretion from both T cell subsets, we next assessed their relative capacity to process Ag.

First, we analyzed the proliferation of TCR transgenic Th1 and Th2 cells in response to native OVA presented by CNS APC or by spleen cells. In the presence of 3 μM native OVA, no T cell proliferation is induced by unstimulated microglia or astrocytes (Fig. 6). IFN-γ-treated microglia stimulate the proliferation of Th1 and Th2 cells at levels similar to those induced by 30- to 100-fold higher numbers of spleen cells. In contrast, astrocytes present native OVA very inefficiently to stimulate proliferation of either Th1 or Th2 cells. In some experiments, little proliferation of Th1 cells is only observed in response to OVA presented by 3 × 10^4 astrocytes. The decreased proliferative T cell responses with increasing microglia density could be due to noxious mediators released by activated microglia and/or could result from activation-induced T cell death (30, 61).

When APC are pulsed with OVA 323–339 peptide (0.1 μM), the pattern of Th1 and Th2 proliferation induced by microglia is
microglia or astrocytes, or spleen cells), in the presence of 3 μM.

The proliferative response is abrogated by addition of anti-I-A d

similarly. Unlike microglia, astrocytes are very inefficient in Ag

process can also be induced by OVA 323–339 peptide. Optimal proliferative responses of Th1 and similar to that observed with native OVA (Fig. 6). Also, astrocytes become able to stimulate T cell proliferation in the presence of OVA 323–339 peptide. Optimal proliferative responses of Th1 and Th2 cells are induced by 3 × 10^8 IFN-γ-treated astrocytes and are similar to or higher than those induced by microglia. However, at lower cell densities (<1 × 10^5), astrocytes are less efficient than microglia in stimulating proliferation of both Th1 and Th2 cells. The proliferative response is abrogated by addition of anti-I-A^d mAb to cultures, and no T cell proliferation occurs in the absence of added Ag or APC (not shown). In conclusion, both microglia and astrocytes can present OVA peptide and induce proliferation of Th1 and Th2 cells, whereas only microglia are able to process and present native OVA leading to activation of both T cell subsets.

When examining T cell cytokine secretion, IFN-γ-treated microglia are the most efficient APC in processing OVA, leading to activation of both Th1 and Th2 cells (Fig. 7). Compared with spleen cells, 30- to 300-fold less microglia are required to induce the same level of IL-2, IFN-γ, or IL-4 production. Consistent with data for proliferative assays, IFN-γ-treated astrocytes process OVA very inefficiently because a very modest production of IFN-γ, but not of IL-2, by Th1 cells and of IL-4 by Th2 cells is observed only at the highest cell density tested (1 × 10^5 cells). In the presence of OVA 323–339 (0.3 μM), unstimulated microglia and spleen cells induce similar Th1 activation, whereas IFN-γ-treated microglia and spleen cells induce similar Th1 activation, whereas IFN-γ-treated astrocytes and spleen cells induce little or no Th cell cytokine secretion, whereas at higher cell densities (>3 × 10^5) microglia do not support T cell proliferation. This suggests that products of activated microglia may negatively affect T cell survival/proliferation or that T cells undergo apoptosis in response to Ag presentation by microglia, as shown in two recent reports (30, 62). We are currently evaluating these two possibilities. These findings also suggest that in vivo restimulation of Th1 and Th2 cells by microglia may result in either T cell death or in further expansion of the differentiated T cell subsets depending on the number of activated microglia and on the strength of the T cell-activating stimuli.

Optimal Th1 proliferation and cytokine production usually require costimulatory signals (e.g., B7 and IL-12) that synergize with each other (16). In our experimental system, MHC class II expression by microglia is critical to elicit T cell cytokine secretion, whereas adhesion/costimulatory molecules, such as ICAM-1, LPS, or phagocytosis can process and present Ag to unprimed and primed T cells, leading to their proliferation (23, 24, 28, 29). In a recent ex vivo study, Ford et al. (30) showed that microglia from the adult rat brain stimulate production of IFN-γ and TNF-α by long-term Ag-specific T cell lines but fail to induce either IL-2 production or T cell proliferation. Our results provide further insights into microglial APC function by showing that microglia can effectively restimulate both Th1 and Th2 cells. Microglia efficiently present synthetic OVA peptide as well as peptide derived from processing of native OVA to both T cell subsets, leading to T cell proliferation and to secretion of IL-2 and IFN-γ by Th1 cells and of IL-4 by Th2 cells. In our culture conditions, unstimulated microglia constitutively express ICAM-1, but little or undetectable MHC class II, B7, or CD40, and are able to induce some degree of Th1 and little Th2 cytokine secretion, suggesting that low levels of MHC class II or other costimulatory signals are sufficient to restimulate T cell effector functions. Pretreatment of microglia with IFN-γ, which up-regulates MHC class II, ICAM-1, and CD40 but neither B7-1 nor B7-2, greatly enhances the capacity of this cell type to stimulate Th1 and Th2 cytokine production and to induce proliferation of both T cell subsets. Interestingly, optimal T cell proliferation is induced by low microglia numbers that induce little or no T cell cytokine secretion, whereas at higher cell densities (>3 × 10^5) microglia do not support T cell proliferation. This suggests that products of activated microglia may negatively affect T cell survival/proliferation or that T cells undergo apoptosis in response to Ag presentation by microglia, as shown in two recent reports (30, 62). We are currently evaluating these two possibilities. These findings also suggest that in vivo restimulation of Th1 and Th2 cells by microglia may result in either T cell death or in further expansion of the differentiated T cell subsets depending on the number of activated microglia and on the strength of the T cell-activating stimuli.
B7-1, and B7-2 do not appear to be involved. Blockade of CD40L on Th1 cells partially reduces IFN-\(\gamma\) production, suggesting that CD40-CD40L interactions contribute to Th1 activation by microglia. Similarly to what was observed in macrophages and dendritic cells (63, 64), ligation of CD40 on microglia may induce IL-12 secretion, which in turn promotes cytokine production by Th1 cells (16, 65). This possibility is currently under investigation. The observation that IFN-\(\gamma\)/LPS-stimulated microglia (that express high levels of ICAM-1, CD40, and B7-2 but are MHC class II negative) are unable to induce T cell activation further supports a major role for MHC class II, rather than adhesion/costimulatory molecules, in the activation of differentiated Th1 and Th2 cells. However, it is possible that IFN-\(\gamma\)/LPS-activated microglia secrete products that have adverse effects on T cells and may therefore inhibit the positive effects of other costimulatory signals.

Astrocytes are also able to restimulate Th1 and Th2 cells and, similarly to microglia, IFN-\(\gamma\) is the most effective stimulus that enables astrocytes to induce T cell proliferation and cytokine secretion. Compared with microglia, IFN-\(\gamma\)-activated astrocytes are less powerful activators of Th1 responses, but are similarly effective in presenting antigenic peptide to Th2 cells leading to IL-4 secretion. The present study also shows that astrocytes have a much lower capacity to process Ag than microglia, as they stimulate some Th1 and Th2 cytokine production only in the presence of very high doses of native OVA. Inefficient Ag processing by astrocytes is confirmed by proliferation studies. Although high numbers of astrocytes (\(\geq 1 \times 10^5\)) induce higher T cell proliferation than microglia to OVA 323–339, at lower cell densities microglia are clearly more efficient than astrocytes in stimulating the proliferation of both Th1 and Th2 cells. The present data are in

**FIGURE 7.** Relative efficiency of microglia, astrocytes, and spleen cells in processing native OVA and presenting OVA peptide 323–339 to induce cytokine secretion by Th1 and Th2 cells. Five \(\times 10^4\) Th1 and Th2 DO11.10 transgenic T cells were cultured together with graded numbers of unstimulated or IFN-\(\gamma\)-treated microglia or astrocytes, or spleen cells, in the presence of 10 \(\mu\)M native OVA or 0.3 \(\mu\)M OVA peptide 323–339. After 24 h, supernatants from triplicate cultures were pooled and cytokines were measured by two-site ELISA assays. Values are means \(\pm\) SEM from three independent experiments.

**FIGURE 8.** Cytokine secretion by Th1 and Th2 cells in response to different doses of native OVA presented by microglia and astrocytes. Five \(\times 10^4\) Th1 and Th2 DO11.10 transgenic T cells were cultured together with 3 \(\times 10^4\) IFN-\(\gamma\)-treated microglia or astrocytes in the presence of increasing amounts of native OVA. After 24 h, supernatants from duplicate cultures were pooled and cytokines were measured by two-site ELISA assays. The data shown are from a representative experiment of three performed.
agreement with the work of Matsumoto, Ohmori, and Fujiwara (29), who reported that microglia, but not astrocytes, are able to present native myelin basic protein and to stimulate T cell proliferation. They are, however, in contrast with several previous reports showing that astrocytes are able to process and present protein Ag (including OVA) to T cell lines, leading to their proliferation (41, 46, 47, 62). A likely explanation for these conflicting data is the degree of contamination of astrocyte cultures by microglia. In fact, as shown herein, even very small amounts of microglia (1–3 × 10^3 cells/well) are sufficient to trigger T cell proliferation. The presence of peptides in the protein preparations used as Ag in previous experiments should also be considered.

As for microglia, astrocyte-induced cytokine secretion by TCR transgenic Th1 and Th2 cells is totally dependent on MHC class II expression and does not appear to involve ICAM-1, which is highly expressed on these cells. We also show that astrocytes cannot be induced to express B7-1 (CD80) or B7-2 (CD86), as already reported (25, 33, 48), or CD40, and that these molecules do not contribute to either Th1 or Th2 activation by astrocytes. In contrast, Nikcevich et al. (47) have recently reported that B7-1 and B7-2 are induced by IFN-γ on mouse astrocytes and are involved in the activation of naive T cells as well as differentiated Th1 cells from the same TCR transgenic mice used in the present study. Neither mouse strain differences nor the type of inducing stimuli can explain these conflicting results, which could be mainly due to contaminating microglia in astrocyte cultures.

The present report is the first one clearly establishing differences between microglia and astrocytes in their Ag-processing capacity and in their efficiency to restimulate distinct T cell subsets. It is likely that the phagocytic properties of microglia, which are functionally similar to macrophages, contribute to the more efficient Ag processing. The higher efficiency of microglia in restimulating Th1 cytokine secretion compared with astrocytes is consistent with the finding that microglia, but not astrocytes, secrete IL-12 (26, 27). IFN-γ-activated microglia are also more potent than spleen cells in Ag processing and in the restimulation of Th1 and Th2 cytokine production. To further define the relative position of astrocytes and microglia in a hierarchy of APC, future studies will compare the function of brain and lymphoid (dendritic cells, B cells) APC.

The present data suggest that microglia, when activated in vivo by IFN-γ and, possibly, by other stimuli that enhance MHC class II expression (e.g., factors released during neuronal damage) (21, 22), may act as powerful APC for the restimulation of peripherally activated Th1 and Th2 cells that have entered the CNS as a consequence of a viral infection, or in inflammatory CNS diseases such as MS (5, 6). In MS, MHC class II, ICAM-1, B7, and CD40 molecules are expressed on activated microglia present within or around white matter inflammatory lesions (25, 31–33), suggesting that these cells can effectively present Ag to infiltrating T lymphocytes. Th1 responses are thought to be involved in the generation of MS lesions (5, 15), although both Th1 and Th2 cytokines have been detected in the brain parenchyma and cerebrospinal fluid at different stages of the disease, indicating disregulation of both cellular and humoral immune responses (31, 66). Since Th2 responses could inhibit inflammation and limit the noxious effects of Th1-mediated immunity (7, 9), the capacity of microglia to activate both T cell subsets may contribute to the pattern of recurrent cerebral inflammation characteristic of MS (67).

Similarly to other nonprofessional APC (e.g., epithelial keratinocytes, endothelial cells) (68, 69), cultured astrocytes appear to be very efficient in Th2 restimulation and are less powerful stimulators of Th1 responses. Despite efficient T cell stimulation demonstrated in vitro, astrocytes express little or no MHC class II molecules in most CNS pathologic conditions (38, 70, 71), suggesting that in vivo Ag presentation by astrocytes may be the exception rather than the rule. However, intrathelial injection of IFN-γ in rats induces a delayed expression of MHC class II molecules on astrocytes (39) and MHC class II molecules are present on reactive astrocytes in MS lesions (35, 36). The low efficiency of astrocytes in processing native protein does not exclude the possibility that astrocytes present Ag at sites of brain inflammation. In MS lesions, the degradation of myelin by proteolytic enzymes may lead to the generation of peptide fragments that could be presented without further processing. Thus, in some circumstances (e.g., during chronic inflammation), astrocytes could present Ag, inducing mainly restimulation of Th2 responses. Previous studies have indicated a role for astrocytes in down-regulating T cell responses through soluble factors that inhibit Ag presentation by other APC (44, 45) and IL-12 production by microglia (27). This inhibitory activity and the capacity of astrocytes to activate Th2 responses may represent important homeostatic mechanisms during recovery from Th1-mediated inflammation.

In conclusion, our data demonstrate that microglia can act as very efficient APC in the CNS by restimulating both Th1 and Th2 responses. Conversely, astrocytes preferentially restimulate Th2 cells. All together, these data suggest that microglia and astrocytes may play distinct roles in the regulation of immune responses against pathogens and in immune disregulation leading to autoimmune attack of CNS-related components.

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