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Astrocytes protect the CNS: antigen-specific T helper cell responses are inhibited by astrocyte-induced upregulation of CTLA-4 (CD152)

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Abstract Astrocytes are the first cells that are encountered by T cells invading the central nervous system (CNS) by crossing the blood-brain barrier. We show that primary astrocytes contribute to the immune privilege of the CNS by suppressing Th1 and Th2 cell activation, proliferation and effector function. Moreover, this astrocyte-mediated inhibition of Th effector cells was effective on already activated, proliferating cells. Transforming growth factor (TGF)- β secreted by astrocytes or T cells was not the major factor in the inhibition. The inhibition of T-cell proliferation induced by astrocytes was mainly mediated by upregulation of CTLA-4 on already activated T cells, which occurred both with and without cell-cell contact. Upregulation of the inhibitory molecule CTLA-4 on autoreactive Th cells, as mediated by astrocytes, thus represents a novel mechanism for securing the immune privilege of the CNS.

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Keywords Transgenic mice · Lymphocyte activation · Neuroimmunomodulation · Immune privilege

Introduction

The CNS is an immunologically privileged site where neurons are protected from damage by inflammation. Within the CNS, astrocytes are the most abundant cells, whose main task is to maintain the physiological homeostasis of neurons. Recently astrocytes have been shown to regulate synaptic activity and neuronal circuitry [1, 2]. They can also have a detrimental effect, notably in the

neuroinflammatory diseases such as multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE). On the other hand, astrocytes can produce a range of neurotrophins which have been shown to be neuroprotective in EAE [3, 4], dementia of the Alzheimer type [5], and Parkinson's disease [6]. Interestingly, astrocytes can act as antigen-presenting cells (APCs) during inflammation by expressing major histocompatibility complex-II (MHC-II) and B7 molecules. While microglia express MHC-II readily upon activation in vivo and in vitro, astrocytic MHC-II expression is seen only during prolonged inflammation in vivo [7] or stimulation by interferon- γ (IFN- γ) in vitro [8]. Regarding their antigen-processing ability, heterogeneous data have been published. While stimulated B10.PL and SJL/J astrocytes were able to process and present native myelin basic protein (MBP) and proteolipid protein, respectively [9, 10], stimulated BALB/c astrocytes lacked the ability to process ovalbumin [11]. Although both microglia and astrocytes are able to produce pro- and anti-inflammatory mediators, there are differences between these two cell types which suggest an anti-inflammatory role for astrocytes [12]. Unlike microglia, astrocytes are not only unable to produce IL-12, which is considered the main factor in Th1 differentiation, but rather, they inhibit microglial IL-12-production [13]. In addition, they produce prostaglandin-E₂ which downregulates microglial activation [13] in response to inflammatory stimuli such as IL-1 β , and TNF- α [14, 15]. Functioning as APCs, murine astrocytes have been shown to stimulate differentiated T cells, and interestingly, Th2 cells more efficiently than Th1 cells [11]. In contrast, while they are able to present protein antigens to CD4⁺ T cells, cytokine-treated human astrocytes did not induce antigen-dependent T-cell proliferation but rather inhibited it [16, 17]. This supports the notion that astrocytes may play an anti-inflammatory role during T-cell-mediated neuroinflammation.

Activation, proliferation and effector function of CD4⁺ Th cells are strictly regulated by signals transduced at the "immunological synapse" between the T cell and the APC, via TCR-MHC-II engagement and additional signaling through costimulatory molecules. The primary costimulatory molecule CD28 and its homologue cytotoxic T lymphocyte-associated antigen-4 (CTLA-4, CD152) on T cells engage the same ligands, B7-1 (CD80) and B7-2 (CD86) on APCs, but CTLA-4 binds with 10- to 100-fold higher affinity than CD28 [18, 19]. CD28 signaling sustains and enhances T-cell activation while CTLA-4 provides inhibitory signals leading to inhibition of T-cell activation and attenuation of ongoing responses [18, 19, 20]. The importance of the inhibition of T-cell responses by CTLA-4 is supported by genetic inactivation of CTLA-4 in mice, which leads to lymphoproliferative disease and early death [18, 19, 21]. In contrast to CD28, which is constitutively expressed on T cells, CTLA-4 is not detectable on resting T cells [18, 19]. Expression of CTLA-4 on the T-cell surface is induced upon activation and peaks at 48h–72 h after the onset of T-cell activation [18, 19]. Thus, it is conceivable that B7/CD28 and B7/

CTLA-4 ligation predominate at different stages of an immune response.

CTLA-4 cross-linking during T-cell activation prevents lipid raft formation [22], thus inhibiting T-cell activation. IL-2 transcription is inhibited, as NFAT, the major transcription factor involved in the activation of IL-2 mRNA synthesis, does not translocate to the nucleus [20]. During optimal T-cell stimulation, at the peak of CTLA-4 expression, the main function of CTLA-4 is to attenuate the T-cell response without inducing apoptosis [19]. Induction of TGF- β production may play a part [23], although this is controversial [24]. CTLA-4 contributes to the maintenance of peripheral tolerance and prevention of autoimmune responses such as EAE [25, 26]. That CTLA-4 is clinically relevant is evidenced by reports that link polymorphisms in the CTLA-4 gene to susceptibility to MS [27, 28, 29, 30, 31, 32, 33]. Thus, although CTLA-4 evidently plays a fundamental role in neuroinflammatory diseases, little is known about its mode of action in the CNS.

Therefore, in this study, we explored the interaction between astrocytes and T cells, paying particular attention to T-cell proliferation and the production of cytokines. We tested the hypothesis that astrocytes can modulate T-cell responses by inducing upregulation of the inhibitory molecules TGF- β and CTLA-4, and that this may contribute to the immune privilege of the CNS.

Materials and methods

Preparation of primary astrocyte cultures

Primary cortical astrocytes were isolated from neonatal (p0-p1) B10.PL mice. The frontal cortex was isolated from the brain, pools of tissue were prepared and then mechanically dissociated through a Nitex membrane (80 μ m) (Fytlis, Neuenburg, Germany). The cells were seeded into poly-L-lysine (10 μ g/ml in H₂O; Sigma-Aldrich, Taufkirchen, Germany) coated plastic dishes (2 brains per 6/24-well plate) and cultured in medium consisting of DMEM (Gibco BRL, Karlsruhe, Germany), plus 10% fetal bovine serum (Gibco), 100 U/ml penicillin/ 100 μ g/ml streptomycin (Biochrom, Berlin, Germany), and 2.5 mM L-glutamine (Gibco). The medium was changed after a day to reduce the number of contaminating microglia, and thereafter every 3–4 days. T cells were added when the astrocytes had formed a confluent monolayer 12–14 days later. The astrocytes were >96% pure as determined by staining for microglia using *Bandereia simplicifolia* isolectin-B₄-FITC staining (Sigma) for fluorescence microscopy, (Fig. 1a) and by rat anti-mouse CD11b-PE (BD Pharmingen, Heidelberg, Germany) staining for flow cytometry (Fig. 1b). Activation of astrocytes was done by addition of 100 U/ml IFN- γ (PeproTech/TEBU, Frankfurt/Main, Germany) and wells were washed prior to addition of T cells.

T-cell polarization

Transgenic mice, carrying a T-cell receptor (TCR) for MBP peptide Ac1-11 on a B10.PL background were a gift of David Wraith, Bristol [34]. Back-crossing was continued, typing the transgene by flow cytometry after double staining for CD4 and V β 8.1/2 (BD Pharmingen). Spleen and lymph node cell suspensions of 8-week-old transgenic mice were washed, erythrocytes lysed, and the cells (2 \times 10⁶ cells/ml) cultured with 6 μ g/ml MBP peptide Ac1-11 (Affina Immuntechnik, Berlin, Germany) in RPMI-1640 (Gibco)

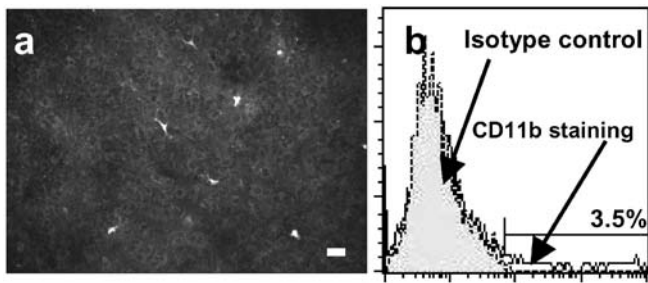


Fig. 1a, b Purity of astrocyte cultures determined by staining of microglial cells. Primary astrocytes were prepared from neonatal B10.PL mice as described in Materials and Methods. Astrocyte cultures were routinely 96–98% free of microglia cells as assessed by immunofluorescence staining of microglia by *Bandeirea simplicifolia* isolectin B₄-FITC (**a**), scale bar=80 μ m; and flow cytometry (**b**). Filled curve, isotype control staining; white curve, CD11b-PE staining. M1 shows the percentage of CD11b⁺ cells of all cells of the astrocyte culture

supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin/100 μ g/ml streptomycin (Biochrom), 2 mM L-glutamine (Gibco), and 50 μ M 2-ME (Merck, Darmstadt, Germany). Either 1 ng/ml mouse rIL-12 (PeproTech/TEBU) plus 1 μ g/ml anti-mouse IL-4 (BD Pharmingen) was added for Th1 cells, or 200 U/ml mouse rIL-4 (PeproTech/TEBU) plus 1 μ g/ml anti-mouse IL-12 (BD Pharmingen) for Th2 cells. After 3 days the cells were expanded in medium containing 100 U/ml of human rIL-2 (Chiron, Ratingen, Germany). Two days after restimulation the differential induction was checked by IFN- γ and IL-4 ELISAs.

T-cell-astrocyte co-cultures for determination of CTLA-4 expression

After 6 days, T cells were restimulated at 10^6 cells/ml with 6 μ g/ml MBP peptide plus irradiated splenocytes of B10.PL mice as antigen-presenting cells. This was done in the presence of either IL-12 for the expansion of Th1 cells or IL-4 for the expansion of Th2 cells. After 24 h of restimulation CD4⁺ T cells were isolated by high gradient magnetic-activated cell sorting. The cells were labeled with superparamagnetic microparticles conjugated to monoclonal antibodies (anti-CD4 MACS beads; Miltenyi Biotec GmbH; Bergisch-Gladbach, Germany) and then separated on a MidiMACS column. FACS analysis showed >95% CD4⁺ cells. The purified Th1 and Th2 cells were then either co-cultured with astrocytes directly or separated by a membrane (pore size: 0.4 μ m, BD Falcon, Heidelberg, Germany) in the presence of MBP peptide at 3.3×10^3 cells/ml. Control Th1 and Th2 cells were cultured without astrocytes. Twenty-four hours later, T cells were collected and fixed in 2% paraformaldehyde for 10 min. Supernatants were stored for further analysis by ELISA. For other experiments, T cells were restimulated with APCs and MBP at day 6 in the presence of astrocytes or seeded onto astrocytes without APCs or MBP peptide. T cells were collected after 48 h and fixed.

FACS analysis of CTLA-4 expression

T cells were stained intracellularly with anti-CTLA-4 (clone: UC10-4F10-11, BD), followed by anti-hamster IgG-Cy5 (from the DRFZ), both at 1 μ g/ml in saponin buffer (PBS/0.5% BSA/0.5% saponin, Sigma) for 15 min at 4°C. Flow cytometry analysis was performed on transgenic T cells stained as above on a FACSCalibur flow cytometer (Becton Dickinson, Mountainview, USA).

Preparation of anti-CTLA-4 and isotype control Fab fragments

Anti-CTLA-4 and control antibodies were purified from supernatants of the hybridoma ATCC: UC10.4F10.11 using protein G columns. The specificity of anti-CTLA-4 antibodies was checked by FACS staining. The anti-CTLA-4 antibodies and Fab fragments were controlled using HPLC analysis, which routinely showed only one peak. Neutralizing anti-CTLA-4 and isotype control antibody Fab fragments were prepared from whole antibodies with the Immunopure Fab preparation kit (Pierce/Perbio Deutschland, Bonn, Germany) and were used at 200 μ g/ml. The toxicity and function of the Fab fragments were tested in antigen-stimulated T-cell cultures using Trypan Blue and CFSE stainings.

Proliferation of T cells in co-cultures with astrocytes

After 6 days of culture, Th1 and Th2 cells were labeled with CFSE [10 μ M 5(6)-carboxyfluorescein diacetate succinimidyl ester, mixed isomers; Molecular Probes/MoBiTec, Göttingen, Germany]. APCs were prepared as for the CD4-depleted splenocytes of B10.PL mice. Th1 or Th2 cells (1×10^6 cells/ml) were co-cultured with astrocytes plus APCs (0.5×10^6 cells/ml) in the presence of 6 μ g/ml MBP peptide. Some of the T cells were pre-incubated for 30 min on ice prior to addition to astrocyte/APC cultures together with 200 μ g/ml anti-CTLA-4 IgG or Fab fragments or isotype control antibodies. Anti-TGF- β 1,2,3 antibody (100 μ g/ml; R&D Systems, Wiesbaden, Germany) or human rIL-2 (50 U/ml; Chiron) were added to some wells. T cells were collected from wells at 24 h, 48 h and 72 h, stained with anti-CD4-Cy5 (1 μ g/ml, 10 min at room temperature) and fixed in 2% paraformaldehyde in PBS for 10 min. T-cell proliferation was analyzed by flow cytometry of CFSE-labeled CD4⁺ T cells. In some experiments the metaphase/anaphase transition inhibitor paclitaxel [35] (ICN Biomedicals, Eschwege, Germany) was used at a concentration of 200 nM. Supernatants were collected and stored frozen until further analysis by ELISA.

T cell-astrocyte co-cultures for the cytometric determination of surface molecules

Th cell-astrocyte co-cultures were set up as for the proliferation assays except for the CFSE labeling, which was omitted. T cells were collected from the wells after 48 h. Supernatants were collected and stored frozen until further analysis by ELISA. Cells were double-stained for CD4 and one of the surface molecules CD25, as well as V β 8.1/8.2 (1 μ g/ml; clones PC61 and MR5-2 respectively, from BD Pharmingen) in PBS/0.5% BSA/0.01% NaN₃ for 10 min at room temperature. Cells were subsequently fixed in 2% paraformaldehyde for 10 min. After washing, staining was analyzed by flow cytometry as described above.

Cytokine ELISAs

Cytokine levels in the 24 h, 48 h and 72 h supernatants of T cells and T cell-astrocyte co-cultures were determined by ELISAs (OptEIA ELISA kits, BD Pharmingen).

Results

Th1 and Th2 proliferation is inhibited in the presence of astrocytes

To address the question of how astrocytes influence Th cell proliferation, we used a co-culture setup and CFSE-labeled Th cells. CFSE labeling of cells allows the detection of both the percentage of cells which have

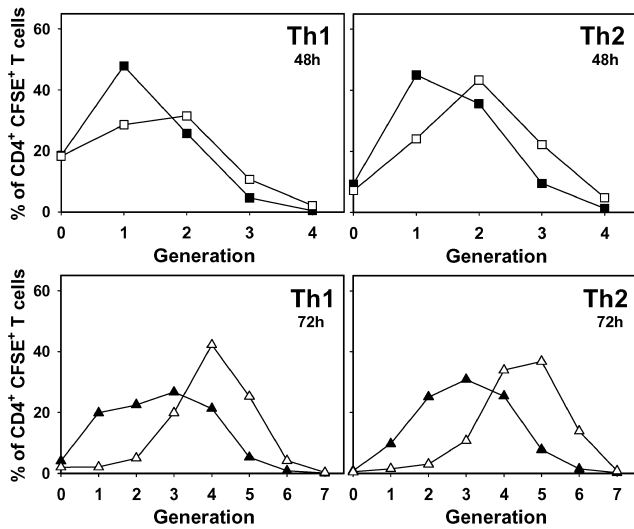


Fig. 2 Astrocytes inhibit T-cell proliferation. Th1 (left panels) or Th2 cells (right panels) were CFSE-labeled and restimulated with APCs and MBP peptide on day 6 after priming in the presence (filled symbols) or absence (open symbols) of astrocytes. T-cell proliferation was assessed by cytometric analysis of the percentage of CD4⁺ cells in different generations [analyzed by sequential halving of the fluorescence intensities (CFSE) with each cell division] after 48 (top panels, squares) or 72 h (lower panels, triangles). Data shown are representative of six independent experiments

divided and the number of generations over a given cell culture period, as the fluorescence intensity is halved by each cell division [36]. A significant reduction was observed in the proliferation of both Th1 and Th2 cells in co-cultures with astrocytes as compared to co-cultures without astrocytes at 48 h, which became even more striking after 72 h (Fig. 2). Interestingly, astrocytes exerted a similar proliferation-inhibiting effect even when T cells and APCs were removed from the astrocyte–T-cell co-cultures after 24 h and were cultured with MBP peptide alone (data not shown). This indicates a rapid and sustained effect on T-cell function. The observed reduction in T-cell proliferation in the CFSE assay was not due to reduced numbers of T cells as a result of T-cell apoptosis, as we analyzed only the living CFSE⁺ CD4⁺ T cells.

Astrocytes alter cytokine secretion and/or consumption by stimulated Th cells

Apart from proliferation, it is possible that the interaction of T cells and astrocytes may influence the production of pro- and anti-inflammatory cytokines. Therefore, we compared the accumulation of cytokines in the culture supernatants of antigen-restimulated Th cells grown in the presence and absence of astrocytes. While IL-2 accumulation in the supernatants of all cultures was equal after 24 h (data not shown), we found marked reductions in all co-cultures with astrocytes as compared to control cul-

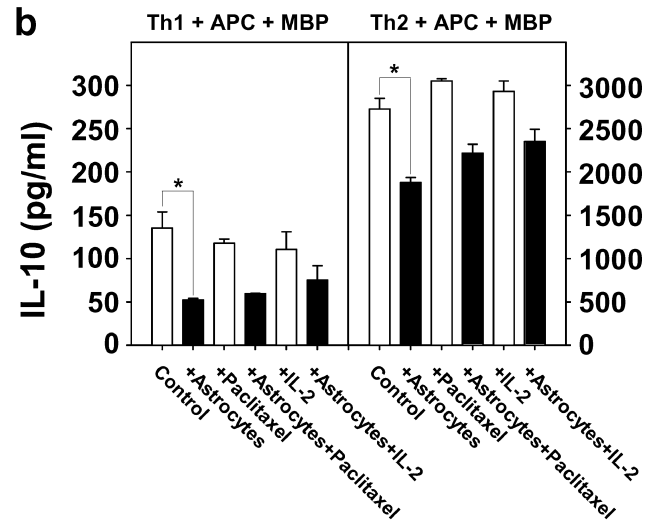
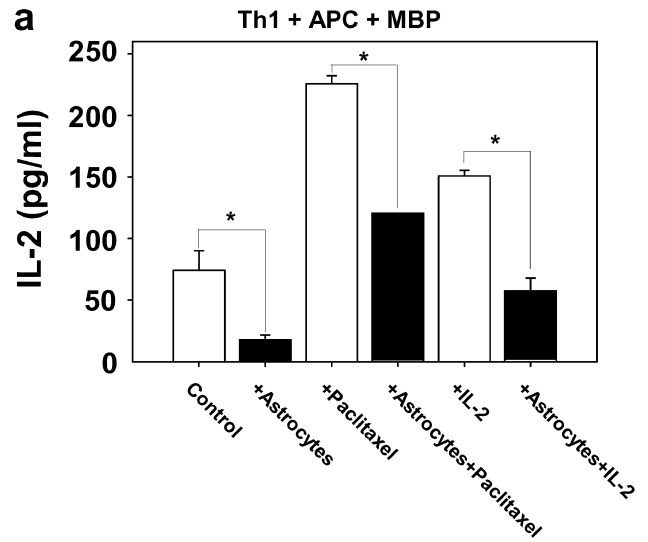


Fig. 3a, b Astrocytes alter cytokine expression of Th1 and Th2 effector cells. Th1 and Th2 cells were restimulated on day 6 with APCs and MBP peptide in the presence (black bars) or absence (white bars) of astrocytes. Supernatants were collected 48 h after the onset of restimulation and analyzed by ELISA for IL-2 [(a) Th1 cells], and IL-10 [(b) left panel Th1 cells, right panel Th2 cells]. Student's paired *t*-test was used for statistical analysis of four independent experiments (* $P < 0.05$)

tures at 48 h after restimulation (Fig. 3a). To exclude proliferation-related effects such as increased cell number and consumption of IL-2 by proliferating cells, we added the cell cycle inhibitor paclitaxel to the co-cultures. In the presence of paclitaxel we found about twice as much IL-2 as in the cultures without added inhibitor, suggesting a consumption of IL-2 by proliferating cells in the absence of paclitaxel. The supernatants of stimulated T cells co-cultured with astrocytes contained significantly less IL-2 than those of stimulated T cells cultured without astrocytes. Addition of recombinant human IL-2 (which is not detectable by our ELISA) led to increased IL-2 produc-

tion by the Th cells, indicating that part of the astrocyte-induced blockade can be reversed by IL-2. Since paclitaxel did not reverse the reduction in IL-2 production by T cells cultured in the presence of astrocytes, this effect can not be due to unequal T-cell numbers in the presence and absence of astrocytes. Thus, either the IL-2 production per cell is reduced, or the frequency of IL-2 producers is lower in co-cultures where astrocytes are present.

IL-10 secretion was also significantly reduced in the presence of astrocytes (Fig. 3b). This was also observed when proliferation was inhibited by paclitaxel. Thus, astrocyte-mediated inhibition of IL-10 production by stimulated T cells must be independent of cell expansion. The source of IL-10 was most likely T cells, as we found only very low amounts in Th1 cultures as opposed to high amounts in Th2 cultures, as would be expected for these T-cell subtypes. Only low levels of IL-10 could be detected in control cultures of astrocytes (data not shown). In contrast to IL-10 production, IL-4 production by Th2 cells was not affected (data not shown).

In the presence of astrocytes, we found a slight reduction in the levels of IFN- γ . This effect was proliferation-dependent, i.e. due to a lower T-cell number in astrocyte-T cell co-cultures, as it was absent in the presence of paclitaxel, where the suppression of proliferation ensured equal cell numbers (data not shown).

To address the question of whether astrocytes interfere with Th cell activation only, or are also able to inhibit cytokine production by activated Th cells we used co-cultures of astrocytes and pre-activated Th cells. For this, Th1 and Th2 cells were cultured under polarizing conditions for 6 days, and were then restimulated with APCs and MBP peptide for 24 h. CD4⁺ T cells were isolated and added to IFN- γ -activated astrocytes or APCs in the presence of MBP peptide. In six independent experiments, we found only 48% of IFN- γ production by Th1 cells in the co-cultures with astrocytes ($1,640 \pm 440$ pg/ml) as compared to the cultures with Th1 and APCs plus MBP peptide ($3,440 \pm 40$ pg/ml). In contrast, IL-4 production reached comparable levels in the co-cultures with astrocytes (270 ± 60 pg/ml) and the cultures with APCs plus MBP peptide (240 ± 60 pg/ml). IL-10 production in the Th2-astrocyte co-cultures ($2,020 \pm 1,150$ pg/ml) was significantly lower than in the cultures of Th2 cells and APCs plus MBP peptide ($4,600 \pm 2,700$ pg/ml). Neither IL-1 β , TNF- α , nor marked production of TGF- β was revealed by ELISA (data not shown). Thus, astrocytes also inhibit cytokine production by previously activated T cells.

Astrocytes mediate downregulation of TCR expression and suppress upregulation of CD25 upon T-cell activation

To determine whether astrocytes interfere with other parameters of T-cell activation, we monitored the TCR density and expression of the activation marker and high affinity IL-2 receptor CD25 in 48 h T-cell co-cultures with astrocytes, astrocytes plus APCs, and APCs alone (Fig. 4). In each case MBP peptide was present and the

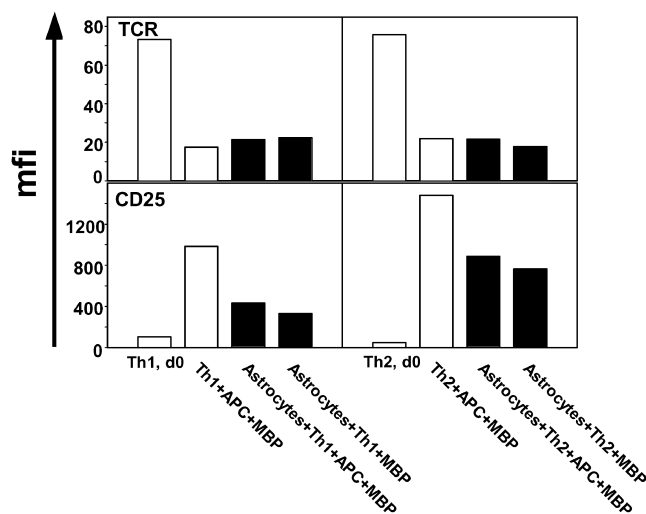


Fig. 4 Astrocytes alter TCR expression and CD25 upregulation of Th1 and Th2 cells. Th1 and Th2 cells were stimulated with MBP peptide for 48 h in the presence (black bars) or absence (white bars) of activated astrocytes and in the presence and absence of APCs as indicated. As controls, non-stimulated Th1 and Th2 cells were stained and fixed (Th1, d0; Th2, d0; left bars). Expression levels of TCR and CD25 on T cells are given as mean fluorescence intensities (mfi) of surface staining monitored by cytometry. Data shown are representative of two independent experiments

astrocytes were activated by IFN- γ to enable antigen presentation. Both Th1 and Th2 cells downregulated their TCR upon contact with astrocytes in the presence and absence of APCs during secondary stimulation. However, stimulation by APCs and MBP peptide in the absence of astrocytes had the same effect. CD25 was upregulated on Th1 and Th2 cells during restimulation, but this effect was notably less in the co-cultures with astrocytes. Differential restimulating capacities of APCs and astrocytes did not appear to play a role, since CD25 expression in the co-cultures with astrocytes was equally low in the presence and the absence of APCs. Thus, we conclude that astrocytes inhibit the upregulation of CD25.

Astrocytes upregulate CTLA-4 protein expression in Th cells

To mimic the invasion of the CNS by activated T cells, previously activated Th1 and Th2 cells were added to activated or non-activated astrocytes in the presence of MBP peptide, and their levels of expression of CTLA-4 were then measured. We detected the total expression of CTLA-4 (surface plus intracellular), as the surface expression is usually very low, but nonetheless well-correlated with intracellular expression [37]. Th1 and Th2 cells both expressed increased CTLA-4 levels after 24 h in co-culture with astrocytes (Fig. 5a and b). This upregulation occurred even in the co-cultures where contact between astrocytes and T cells was prevented by a membrane (Fig. 5a). Moreover, we found that non-

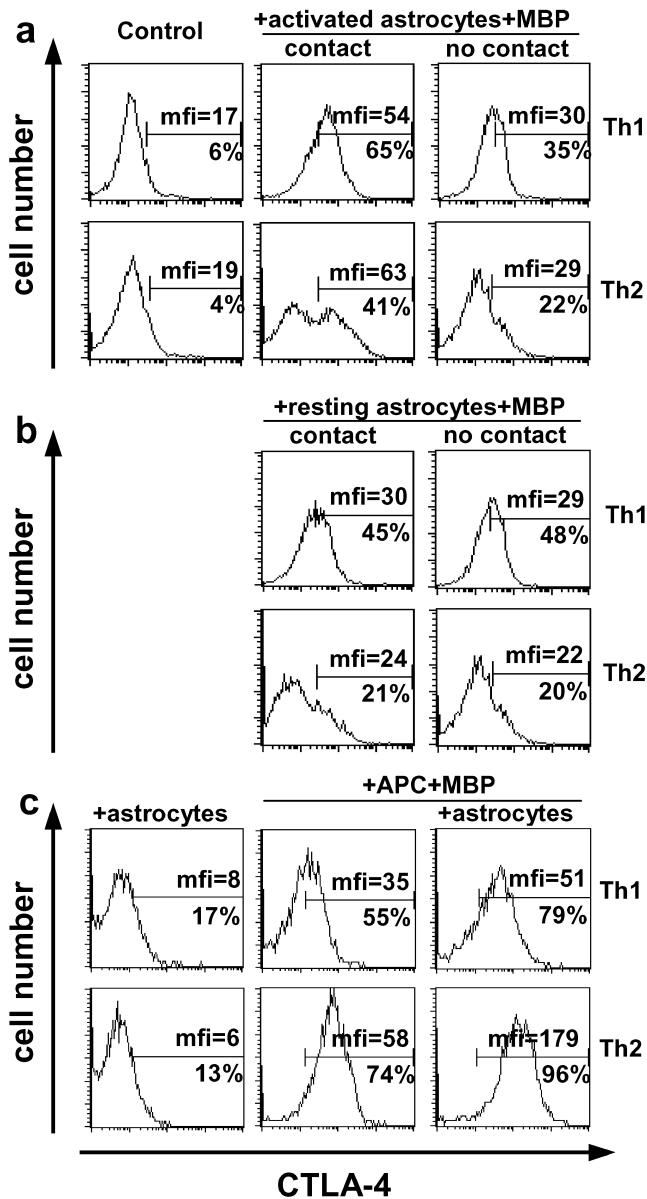


Fig. 5a-c Astrocytes enhance the expression of CTLA-4 on activated Th1 and Th2 cells independent of direct cell-cell contact. Th1 and Th2 cells were stimulated with APCs and MBP peptide for 24 h. The preactivated Th cells were isolated by magnetic activated cell sorting (MACS) and were co-cultured with activated (**a**) or resting astrocytes (**b**) in the presence of MBP peptide. The cells were either co-cultured with astrocytes directly (*middle panel*, “contact”) or separated by a membrane (*right panel*, “no contact”). As controls, Th1 and Th2 cells were cultured in the absence of astrocytes or MBP peptide (*left panel*). **c** Resting Th1 and Th2 cells were seeded out on astrocytes without MBP peptide for 48 h (*left panels*, “+ astrocytes”), or stimulated with APCs and MBP peptide for 48 h in the absence (*middle panels*) or presence (*right panels*) of astrocytes. Expression levels are given as mean fluorescence intensities (mfi) of total CTLA-4 staining of TCR transgenic Th1 and Th2 cells. Indicated percentages of CTLA-4⁺ CD4⁺ cells are corrected for the respective isotype control staining. Data shown are representative of five independent experiments

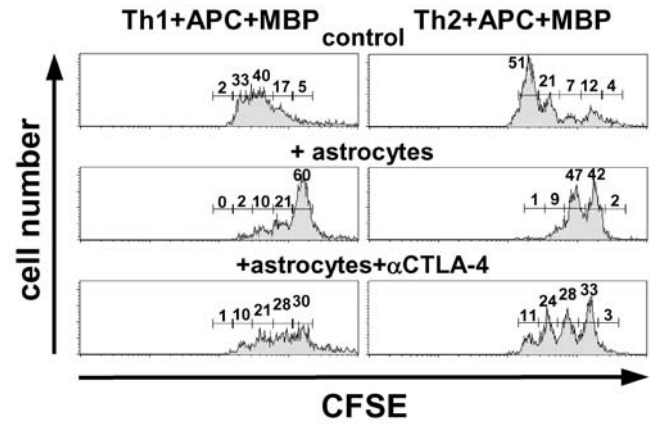


Fig. 6 Serological inactivation of CTLA-4 rescues Th1 and Th2 proliferation in the presence of astrocytes. On day 6 after priming, CFSE-labeled Th1 and Th2 cells were restimulated with APCs, MBP peptide, and astrocytes for 72 h. Neutralizing α CTLA-4 antibody (*lower panels*) or isotype control antibody (*middle panels*) were added to the cultures at 200 μ g/ml. CD4⁺ T-cell proliferation was assessed by cytometric analysis of CFSE intensities. Percentages of cells in each generation relative to the whole population are indicated by numbers. Data shown are representative of four independent experiments

activated astrocytes could also enhance CTLA-4 expression in T cells across a membrane, suggesting that a soluble factor was responsible (Fig. 5b). To investigate whether astrocytes induce CTLA-4 expression independent of TCR-MHC-II interaction, we added resting Th1 or Th2 cells (6 days after primary stimulation) to activated astrocytes in the presence or absence of MBP and APCs for 48 h. Under these conditions, upregulation of CTLA-4 expression did not occur when the MBP was omitted, demonstrating that MHC-II-TCR interaction is a prerequisite for CTLA-4 induction on T cells. More importantly, the upregulation of CTLA-4 in Th cells cultured with APCs and MBP in the presence of astrocytes exceeded the extent of upregulation observed in the absence of astrocytes (Fig. 5c).

Astrocytes inhibit T-cell proliferation mediated by CTLA-4

The addition of IL-2 to the co-cultures rescued Th1 and Th2 cell proliferation only slightly. Only 15% of Th1 cells and 14% of Th2 cells exceeded the level of proliferation of the majority of the astrocyte-inhibited Th cells, showing that the inhibition could not be solely explained by reduced IL-2 production. Alternatively, blockade of the anti-proliferative cytokine TGF- β by anti-TGF- β led to only 8% of Th1 cells and 12% of Th2 cells exceeding the proliferation of the majority of the astrocyte-inhibited Th cells, showing that TGF- β operates only to a minor extent. The functional significance of the increased astrocyte-induced CTLA-4 expression was tested by blocking CTLA-4 with anti-CTLA-4 antibodies or their Fab fragments, with the Fab fragments showing

the same effect as whole antibodies (data not shown). This treatment preserved the proliferation of the majority of both Th1 and Th2 cells in the co-cultures with astrocytes (Fig. 6).

Discussion

Activated T cells are able to cross the blood-brain barrier not only in neuroinflammatory diseases, but also in the healthy brain [38]. We demonstrate that astrocytes, which constitute the blood-brain barrier, could potentially protect the CNS against local inflammatory T-cell responses. Astrocytes proved to be able to attenuate the activation, proliferation, and cytokine production of Th1 and Th2 cells. The inhibition mediated by astrocytes appears to be mainly effected by enhanced CTLA-4 expression of activated T cells. The normally low B7 expression on astrocytes and microglia [8] should favor CTLA-4 over CD28 ligation due to the higher affinity of the former. Thus, the higher affinity of CTLA-4 binding to B7 ensures that T cells become downregulated as soon as CTLA-4 becomes upregulated. Astrocytes will shift the balance further towards B7/CTLA-4 interaction by up-regulating CTLA-4 on T cells as we have shown. In the healthy brain, B7 and MHC-II expression on local APCs such as astrocytes and microglia is very low. Under these conditions, astrocyte-induced CTLA-4 may be sufficient to minimize costimulation and exert inhibitory signals on T cells that have entered the CNS via the blood-brain barrier.

It is known that astrocytes express CD95L, which enables them to induce apoptosis in T cells [39, 40]. Still, T cells encountering astrocytes have to be susceptible to CD95-induced apoptosis which is usually not before day 5 after the onset of stimulation and does not apply to all differentiated T cells. Indeed, non-apoptotic T cells can be detected in the CNS. Thus, other mechanisms, like the induction of inhibitory molecules such as CTLA-4 on reactive T cells, are needed to locally protect the CNS from unwanted immune responses. To date, CTLA-4 upregulation in T cells has been considered as an event subsequent to TCR ligation [19], synergized by CD28 and IL-2 costimulation. Our data suggest that cognate interaction is one mechanism of CTLA-4 induction, while another must be independent of antigen-presentation and even of direct cell-cell contact, suggesting the involvement of a soluble factor. Such a soluble factor would enable astrocytes to instruct even nonadjacent T cells, building up a local anti-inflammatory environment in the CNS. We surmise that the identification of the soluble factor leading to the upregulation of CTLA-4 on recently activated T cells could be therapeutically important. Experiments aimed at identifying the CTLA-4-inducing factor are currently in progress.

It is still a matter of controversy whether astrocytes express MHC-II *in vivo* [8]. However, for this newly described CTLA-4 upregulating effect astrocytic MHC-II expression is not necessary as it occurs in a cell-contact

independent fashion, as well as in co-cultures with resting astrocytes.

While we describe the inhibitory effects astrocytes exert on T cells, others have shown that astrocytes can stimulate T-cell functions [11, 41, 42]. This apparent discrepancy can be explained by different experimental setups. While other studies evaluated the abilities of astrocytes to stimulate T cells by antigen-presentation using astrocytes as antigen-presenting cells, we provided optimal stimulation for T cells by having professional APCs and antigen in the cultures where astrocytes were added. Thus, astrocytic inhibitory or stimulatory effects could be discerned from baseline effects occurring during T cell-APC interaction. By using this approach we also avoided differences in the stimulatory capacity of astrocytes towards Th1 versus Th2 cells [11, 41]. Microglia exhibit a higher efficiency in stimulating T-cell responses than astrocytes, i.e. lower cell numbers are required for stimulation of Th1 but not Th2 cells [11]. However, our astrocyte populations contain routinely less than 4% of microglia which could not have had any substantial effects on our experiments since their antigen-presenting abilities do not exceed that of our added APCs.

Hyporesponsiveness of T cells induced by astrocytes has been described before and was interpreted as being a result of downregulation of the TCR [43] and insufficient stimulation by astrocytes owing to the fact that they express only low amounts of ICAM-1 and MHC-II [44]. Since we could demonstrate TCR downregulation in stimulated T cells irrespective of the presence of astrocytes, we do not consider this a contribution to reduced T-cell proliferation, but rather a feature accompanying T-cell stimulation. In contrast, our finding of CD25 upregulation being suppressed by astrocytes points to incomplete activation of T cells as a cause of hyporesponsiveness. Strikingly, when we stimulated the T cells with professional APCs, we still found T-cell activation to be downregulated in the presence of astrocytes. Therefore, the anti-inflammatory influence of astrocytes on T-cell activation is fundamental, as it not only inhibits T-cell activation, but also overrides optimal T-cell stimulation with professional APCs. Loss of the proliferative capability of infiltrating cells in EAE lesions immediately after entrance into the CNS might also be due to astrocyte-induced CTLA-4 upregulation [45].

We have shown that astrocytes employ CTLA-4 upregulation to downregulate T-cell responses in order to prevent and counteract inflammation in the CNS. For therapeutic purposes, e.g. in tumor therapy, inactivation of CTLA-4 in order to enhance T-cell responses, has been a main strategy so far. However, as we demonstrate in this study, the opposite approach, namely induction of CTLA-4 for prevention of inflammatory responses, should also be considered seriously.

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